Published in final edited form as:

Biochemistry. 2017 August 15; 56(32): 4117–4126. doi:10.1021/acs.biochem.7b00613.

Ribosome Mediated Quinary Interactions Modulate In-Cell Protein Activities

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Abstract

Ribosomes are present inside bacterial cells at micromolar concentrations and occupy up to 20% of the cell volume. Under these conditions, even weak quinary interactions between ribosomes and cytosolic proteins can affect protein activity. By using in-cell and *in vitro* NMR spectroscopy, and biophysical techniques, we show that the enzymes, adenylate kinase and dihydrofolate reductase, and the respective coenzymes, ATP and NADPH, bind to ribosomes with micromolar affinity, and that this interaction suppresses the enzymatic activities of both enzymes. Conversely, thymidylate synthase, which works together with dihydrofolate reductase in the thymidylate synthetic pathway, is activated by ribosomes. We also show that ribosomes impede diffusion of green fluorescent protein *in vitro* and contribute to the decrease in diffusion *in vivo*. These results strongly suggest that ribosome-mediated quinary interactions contribute to the differences between *in vitro* and *in vivo* protein activities and that ribosomes play a previously under-appreciated nontranslational role in regulating cellular biochemistry.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00613.

Purified ribosomes contained no small unknown molecules; ³¹P NMR spectra of the phosphate buffer; DHFR binds to ribosome *in vitro* and in-cell; TS binds to total *E. coli* RNA; ribosome does not bind to Trx; and ribosome binds to EmGFP (PDF)

Author Contributions

C.D., S.M., and S.R. conducted experiments and analyzed the data, A.S. designed the experiments, and A.S., C.D., D.B., and S.R. wrote the paper.

The authors declare no competing financial interest.

The ribosome is a large RNA–protein complex that shares similar architecture in all branches of life. It synthesizes proteins by translating the genetic information residing on mRNA into a specific sequence of amino acids.^{1,2} The ribosome also binds to a large number of cellular proteins unrelated to translation,^{3–5} such as metabolic enzymes, and may play a role in ribosomal biogenesis, transcription activity, DNA repair, and morphological transformations.^{6,7}

In bacteria, the ribosome concentration is linearly proportional to the growth rate⁸ and can reach tens of micromolar concentration in rapidly dividing cells and constitute up to 40% of the total cell dry weight.⁹ At this high concentration, even a weak, micromolar, protein affinity for the ribosome will generate a large intracellular population of bound species that may have physiological relevance by altering protein biochemistry.

NMR spectroscopy has proven to be a reliable technique to identify protein–RNA structural interactions in solution.¹⁰ The in-cell NMR^{11–15} spectra of *E. coli* thioredoxin, Trx, adenylate kinase, ADK, human FK560 binding protein, FKBP, and ubiquitin show that the proteins exhibit large, megadalton, apparent molecular weights due to transient weak, or quinary^{16–18} interactions with components of the cytosol.¹⁹ *In vitro* NMR studies of reconstituted systems have confirmed the likelihood that quinary structure is mediated by protein–RNA interactions.^{19–21} Since rRNAs constitute 90% of all cellular RNA⁹ and are part of functional ribosomes, we hypothesized that quinary structures may be mediated by protein–ribosome interactions. In this study, we describe the linkage between quinary interactions and biological activity by showing that protein–ribosome interactions affect the enzymatic activity, binding, and diffusion of selected targets, the metabolic enzymes ADK, dihydrofolate reductase, DHFR, and thymidylate synthase, TS, and green fluorescent protein, GFP. We suggest that protein–ribosome interactions contribute to the difference between *in vitro* and *in vivo* protein activities.²²

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals used were of molecular biology grade or better.

Plasmid Construction

DNA encoding full-length DHFR was amplified from *E. coli* DNA using oligonucleotides 5′-GGTACCATGATCAGTCTGATTGCGGCG-3′ and 5′-CAGCTCTTTTAATGGCGGCGAGGTCTTAG-3′. The sequence was ligated into pRSF-1b (Novagen) using the *Kpn*I and *Sa*I linker sites. The resulting plasmid, pRSF-DHFR, confers kanamycin resistance and expresses a DHFR from the T7 *lac* promoter. DNA encoding full-length TS was amplified from *E. coli* DNA using oligonucleotides 5′-TTTTTTGGTCTCTGCGCTTAGATAGCCACCGGC-GCTTTAATGC-3′ and 5′-TTAAAAGGTC-TCTAATGCATCACCATCACCATCAC-CTGGAAGTTCTGTTCCAGGGGCCCAA-ACAGTATTTAGAACTGATGCAAAAAGTGCTCG-3′. The sequence was ligated into pASK-3+ (IBA GmbH) using the *Bsa*I linker sites. The resulting plasmid, pASK3+-TS,

confers ampicillin resistance and expresses a fusion protein with an N-terminal His-tag from a tet promoter. pRSET-EmGFP (Invitrogen) was digested using *NheI* and *NcoI* to remove the T7 gene 10 leader, Xpress epitope, and enterokinase recognition site. The resulting plasmid, pRSET-6xHis-EmGFP, confers ampicillin resistance and expresses His-tagged emerald GFP, EmGFP, fusion protein from the T7 *lac* promoter.

Protein Overexpression

Reduced proton density (RED-PRO)-labeled protein samples were prepared as previously described.²³ *E. coli* strain BL21(DE3) codon+ (Novagen) was separately transformed with plasmids pRSF-ADK,¹⁹ pRSF-DHFR, pRSF-Trx,¹⁹ pASK3+-TS, and pRSET-EmGFP. The pRSF plasmids (Novagen) confer resistance to kanamycin, contain the T7 *lac* promoter, and express fusion proteins with an N-terminal His-tag. The pASK plasmid (IBA GmbH) confers resistance to ampicillin and contains the Tet-inducible promoter. Fifty milliliters of LB medium supplemented with 35 µg/mL of kanamycin (pRSF) or 150 µg/mL of carbenicillin (pRSET, pASK3+) was inoculated with a single colony and grown overnight at 37 °C and 250 rpm. The overnight cultures were diluted to an OD₆₀₀ of 0.07 in 500 mL of LB medium containing the appropriate antibiotic. Cells were grown at 37 °C and 250 rpm until the OD₆₀₀ reached 0.9–1.0. Cells were pelleted, washed with M9 medium, and resuspended in 100 mL of deuterated M9 medium supplemented with 1.0 g/L ¹⁵N-ammonium chloride and 0.4% glucose as the sole nitrogen and carbon sources, respectively. The culture was incubated at 37 °C for 20 min, and expression of REDPRO-labeled protein was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside, and expression of pASK3+-TS was induced with 0.2 µg/mL of anhydrotetracycline. Protein expression proceeded for 20–24 h at 37 °C. For in-cell NMR experiments, 50 mL aliquots of the culture with OD₆₀₀ of 1.15 to 1.50 were pelleted, washed twice with NMR buffer, and resuspended in 300 µL of NMR buffer, 10 mM potassium phosphate, 10 mM magnesium acetate, pH 6.5, and 10% D₂O. After each in-cell NMR experiment, the sample was recovered, the cells were sedimented, and a ¹H–¹⁵N HSQC spectrum was collected on the sample supernatant. No protein peaks above noise level were detected, indicating no cell leakage. Overexpression of ¹⁵N labeled protein followed the same procedure as that noted above, with H₂O replacing D₂O in the M9 medium.

Protein Purification

All proteins were purified under native conditions using Ni-NTA (Qiagen) affinity chromatography. Proteins were dialyzed against either NMR buffer or specific assay buffer. The His-tag was not removed from the proteins since it does not bind to RNAs or ribosomes and, thus, does not interfere with the experiments.¹⁹

Ribosome Preparation

Functionally active ribosomes were purified by using a published protocol²⁴ with slight modifications. *E. coli* cells were grown in LB medium to an OD₆₀₀ of 0.5 to 0.7 and resuspended in lysis buffer, 20 mM Tris-HCl, pH 7.2, 100 mM ammonium chloride, 10 mM magnesium chloride, 0.5 mM EDTA, and 6 mM βME, at a density of 1 g of cells per mL before sonicating with a Model 250 Digital Sonifier (Branson). The lysate was centrifuged at 30,000g for 45 min and the supernatant layered onto lysis buffer containing 37.7% sucrose

prior to centrifuging at 280,000g for 18 h at 4 °C in an Optima LE-90K Ultracentrifuge (Beckman Coulter) using a SW41 Ti rotor. The clear pellet was washed four times with wash buffer, 10 mM Tris-HCl, pH 7.4, 1 M ammonium chloride, 10 mM magnesium acetate, and 2.5 mM DTT, to remove residual ATPase activity.²⁴ The ribosome pellet was resuspended in ribosome buffer, 10 mM potassium phosphate, pH 6.5, 10 mM magnesium acetate, and 1 mM DTT. To verify that no unknown small molecules copurified with the ribosomes, 5 mL of 1 μM ribosome solution was concentrated to 4.5 mL by using a 30 000 Da cutoff Amicon Centrifugal Filter Unit (EMD Millipore). ¹H NMR spectra were collected on both the filtrate and the ribosome buffer (Figure S1). The spectra showed no differences between these samples. Concentration was determined by absorbance at 260 nm, using an $\epsilon_{0.1\%} = 15 \text{ mL}/(\text{mg} \times \text{cm})$. Only ribosome solutions with a 260/280 nm ratio of 1.97 to 1.98 were used.

NMR Experiments

Most in-cell and *in vitro* NMR spectra were recorded at 298 K using a 700 MHz Bruker Avance II NMR spectrometer equipped with a TXI cryoprobe. For *in vitro* experiments, all proteins were dissolved in NMR buffer, 10 mM potassium phosphate, pH 6.5, containing 1 mM DTT. ¹⁵N-Edited cross-relaxation-enhanced polarization transfer heteronuclear multiple quantum coherence transverse relaxation-optimized²⁵ (¹H-¹⁵N CRINEPT-HMQC-TROSY) experiments¹⁹ were performed with Watergate water suppression and CRIPT transfer delays of 350, 250, and 200 ms for ADK, Trx, and DHFR, respectively. ¹⁵N-Edited heteronuclear single quantum coherence (¹H-¹⁵N HSQC) experiments,²⁶ were performed with Watergate water suppression to collect the spectra of [*U*-¹⁵N] EmGFP and [*U*-¹⁵N] TS.

¹H-³¹P Carr Purcell Meiboom Gill pulse train heteronuclear single quantum coherence (HP-CPMG-HSQC²⁷) spectra were recorded at 298 K using an Avance III HD 600 MHz spectrometer (Bruker) equipped with a QCI-P cryoprobe (Bruker). AMP-PCP and NADPH were titrated against 2 μM and 1 μM ribosome, respectively, in NMR buffer containing 10 mM magnesium acetate. Total free AMP-PCP and NADPH were measured in the absence of ribosome by integrating the free peak at 2.13 ppm and -2.72 ppm, in the hydrogen and phosphorus dimensions, respectively, for AMP-PCP, and at 3.96 ppm and -12.04 ppm in the hydrogen and phosphorus dimensions, respectively, for NADPH. ³¹P spectra of the NMR samples were collected to monitor for possible changes in pH during the titrations (Figure S2). All experiments are in duplicate.

Bound AMP-PCP and NADPH were calculated by subtracting the free peak volume from the reference total free concentration at each step of the titration. Bound versus free concentrations were analyzed using GraphPad Prism 6 Software and fit to the following equation:

$$Y = B_{\max} \times X/(K_d + X) + NS \times X \quad (1)$$

where *Y* is the concentration of bound ligand, *X* is the concentration of free ligand, *B*_{max} is maximum concentration of specifically bound ligand, *K*_d is the dissociation constant, and *NS* is the slope of nonspecific binding.

Adenylate Kinase Assay

The ADK assay links the generation of ADP to the hydrolysis of NADH.²⁸ ADK was dialyzed into ADK assay buffer, 50 mM Tris-HCl, pH 7.4, 100 mM potassium chloride, 2 mM magnesium chloride, and 10 mM magnesium acetate. The assay was carried out at 15 °C in ADK assay buffer containing 1 mM phosphoenolpyruvate, 0.1 mM NADH, 0.3 mM AMP, and 5 units each of pyruvate kinase and lactate dehydrogenase. The reaction was initiated by adding ATP and monitored at 340 nm using a Lambda 35 ES UV/vis spectrophotometer (PerkinElmer) equipped with a PCB-150 Peltier controlled fluid circulator and a PTP-1 thermostated cell holder. Data were collected at a rate of 10 scans per second over an appropriate time course per reaction. All experiments are in triplicate. Data were analyzed using GraphPad Prism 6 software and fit to the following equation:

$$\nu_o = V_{\max} \times X / (K_M + X \times (1 + X/K_I)) \quad (2)$$

where ν_o is the initial reaction rate, X is the substrate concentration, K_M is the Michaelis–Menten constant, $V_{\max}$ is the maximum enzyme velocity, and K_I is the dissociation constant for substrate binding. K_I corresponds to the concentration required to produce the half-maximum rate.

Dihydrofolate Reductase Assay

DHFR kinetics were determined by following the breakdown of NADPH. DHFR was dialyzed into DHFR assay buffer, 40 mM HEPES, pH 6.8, and 10 mM magnesium acetate, and used at a concentration of 4 nM. The assay was carried out at 25 °C in DHFR assay buffer containing 200 μ M NADPH. The reaction was initiated by adding DHFR and monitored at 340 nm using a Lambda 35 ES UV/vis spectrophotometer (PerkinElmer). All experiments are in triplicate. Data were analyzed using GraphPad Prism 6 software and fit to the following equation:

$$\nu_o = V_{\max} \times X / (K_M + X) \quad (3)$$

Thioredoxin Activity Assay

The disulfide reductase activity of Trx was determined using a turbidimetric insulin precipitation assay.²⁹ Trx and ribosomes were dialyzed into Trx assay buffer, 10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 10 mM magnesium acetate, and 2.5 mM DTT. Trx was used at a concentration of 4 μ M. The assay was carried out in Trx assay buffer at 25 °C. The reaction was initiated by adding insulin to 0.75 mg/mL (0.13 mM) and monitored at 650 nm using a Lambda 35 ES UV/vis spectrophotometer (PerkinElmer). All experiments are in duplicate. Results were imported into OriginPro for analysis.

Thymidylate Synthase Assay

TS kinetics were determined by following the oxidation of 5,10-methylenetetrahydrofolate to 7,8-dihydrofolate,³⁰ which was monitored at 338 nm using a Lambda 35 ES UV/vis

spectrophotometer (PerkinElmer). TS was dialyzed into TS assay buffer, 42 mM Tris-HCl, pH 8.0, 26 mM magnesium chloride, 1.06 mM ethylenediaminetetraacetic acid, and 10 mM magnesium acetate. The assay was carried out at 15 °C with TS assay buffer containing 1 μ M TS, 0.3 mM tetrahydrofolic acid, 15.8 mM formaldehyde, and 106 mM β -mercaptoethanol with and without 0.5 μ M ribosome. Adding dUMP and ribosome, in the absence of TS, resulted in no increase in absorbance at 338 nm. Data were collected at a rate of 10 scans per second over an appropriate time course per reaction. All experiments are in triplicate. Data were analyzed using GraphPad Prism 6 software and fit using eqs 2 and (3).

Fluorescence Titration

ADK and ribosomes were dialyzed extensively against 10 mM potassium phosphate, pH 7.5, and 10 mM magnesium phosphate. Measurements were performed on a Fluorolog-3 fluorescence spectrophotometer (HORIBA Jobin Yvon) at 25 °C in a 0.2 mL quartz cuvette (Starna). ADK was titrated from 0.5 μ M to 50 μ M in 8 steps, using a 500 μ M stock solution, into 0.5 μ M ribosome. Titrations in the absence of the ribosome and in the absence of ADK were performed as references. Tryptophan fluorescence was measured using an excitation wavelength of 285 nm. The fluorescence emission signal was subtracted from the signal of the reference titration and normalized by the maximum fluorescence emission. Differences were adjusted by the dilution factor and plotted against the final concentration of added ADK. All experiments are in triplicate. Curve fitting (OriginLab) was performed to find the best values for K_d using a single-site binding isotherm approximation.

Confocal Microscopy of EmGFP

Images of EmGFP were taken and processed using a Zeiss LSM 710 confocal microscope and Zen software (Carl Zeiss) utilizing a Plan-Apochromat 63 $\times$ /1.40 Oil DIC M27 objective. The image size was 26.99 μ m $\times$ 26.99 μ m (256 $\times$ 256 pixels), and bleaching occurred in a 5 μ m diameter cylinder centered in each image. Photobleaching was accomplished by using an argon laser at 488 nm and 18 mW, and detection was performed between 0.04 and 0.1 mW of total power. Filtering occurred between 493 to 588 nm, and images were scanned every 76 ms in a bidirectional manner. EmGFP was prepared in 90% NMR buffer, 5% glycerol, and 5% SlowFade light antifade reagent (Molecular Probes). Ten microliter samples were spotted onto a slide, covered and sealed with nail polish. Characteristic diffusion times, $\tau_{1/2}$, were calculated by analyzing intensity recovery data. The corresponding ratio of $\tau_{1/2}$ values are equivalent to D/D_0 .³¹ The ratio of diffusion times in the presence and absence of crowders, D/D_0 , is given by

$$\ln(D/D_0) = -(\Delta r/R) \times \xi \quad (4)$$

where

$$\xi = 3\nu_p/(1 - \nu_p) + 9\nu_p^2/(2 \times (1 - \nu_p)^2) + 9\nu_p^3/(4 \times (1 - \nu_p)^3) \quad (5)$$

and ν_p is the volume fraction of biopolymer crowders.³²

RESULTS

ADK Binds to Ribosomes

The ^1H - ^{15}N CRINEPT-HMQC-TROSY²⁵ spectrum of $\sim 300\ \mu\text{M}$ intracellular [U - ^2H , ^{15}N] ADK in *E. coli* shows that the protein engages in quinary interactions by using specific interaction surfaces that consist of both hydrophobic and hydrophilic residues.¹⁹ In contrast, the *in vitro* NMR spectrum of $10\ \mu\text{M}$ [U - ^2H , ^{15}N] ADK is completely and uniformly broadened in the presence of $1\ \text{mg/mL}$ of total RNA.¹⁹ The spectra are characteristic of intermediate exchange between ADK and total RNA implying a micromolar, $\sim 10\ \mu\text{M}$, binding affinity for the interaction.¹⁹ Adding $1\ \mu\text{M}$ ribosome to $10\ \mu\text{M}$ [U - ^2H , ^{15}N] ADK *in vitro* resulted in selective peak broadening and chemical shift changes in the spectrum of ADK that resemble those of the in-cell NMR spectrum (Figure 1A). This result implies that, in contrast to total RNA, ADK specifically interacts with ribosomes. Residues affected by ribosome binding reside in the conserved ADK core domain suggesting that substrate binding may not be affected (Figure 1B/C).

The strength of the interaction between ADK and the ribosome was independently measured by monitoring changes in tryptophan fluorescence due to ADK-ribosome binding. Purified ADK was titrated into $0.5\ \mu\text{M}$ ribosome. An apparent K_d of $3.7 \pm 0.4\ \mu\text{M}$ was resolved consistent with that estimated by using NMR spectroscopy. Because the in-cell concentration of ribosome is in the micromolar range, a substantial fraction of total cellular ADK may be bound to the ribosome under physiological conditions, thus explaining the apparent megadalton size of ADK.¹⁹

ATP Binds to Ribosomes

The in-cell NMR spectrum of ADK is consistent with a nucleotide-free or open configuration.¹⁹ Adding chloramphenicol to the growth medium increases the cellular production of ATP³⁴ and shifts the in-cell NMR spectrum of ADK from that of free to ATP-bound.¹⁹ Since the cellular concentration of ATP is greater than $1\ \text{mM}$ and the affinity of ATP for ADK is $\sim 50\ \mu\text{M}$ *in vitro*,³⁵ the in-cell concentration of free ATP must be a small fraction of the total ATP. We proposed that ATP binding to ribosomes provides one possible mechanism for reducing the concentration of free ATP in *E. coli*, thus maintaining ADK in the open configuration.

ATP binding to ribosomes was measured by using AMP-PCP, a noncleavable ATP analogue. 2D ^1H - ^{31}P -correlation NMR experiments, which show gradual changes in chemical shifts, were performed to quantify bound and unbound species and to estimate the binding affinity. We monitored possible changes in the pH of the titrated solution by observing the ^{31}P chemical shift of the phosphate peak. No change in the ^{31}P chemical shift of the phosphate peak was detected during titration indicating that the pH of the solution remained constant (Figure S2). At ATP concentrations below $\sim 10\ \mu\text{M}$, the binding of AMP-PCP to the ribosome can be fit to a single class of binding sites with an apparent K_d of $6 \pm 2\ \mu\text{M}$; at higher concentrations, the binding was nonspecific (Figure 2A).

Ribosomes Modulate ATP Binding to ADK

To understand how ADK–ribosome interactions affect ATP binding to ADK, the effect of ribosomes on ATP-bound ADK was evaluated *in vitro* by using NMR spectroscopy. Titrating ATP into purified 10 μM [$U\text{-}^{15}\text{N}$] ADK resulted in systematic changes in chemical shifts and peak broadening of interacting residues. As the concentration of ATP was increased from 0 to 40 μM , selected peaks showed systematic changes in chemical shifts and concomitant broadening (Figure 2B). At 80 μM ATP in the presence of 1 μM ribosome, the spectrum of the ATP-bound ribosome–ADK complex resembled that of 40 μM ATP-bound ADK (Figure 2B), suggesting that ATP binding to the ribosome reduced the concentration of free ATP available for binding to ADK.

Protein–Ribosome Interactions Affect ADK Activity

It is important to understand how the ADK–ribosome interaction affects ATP binding to ADK because both ADK and ATP play a critical role in maintaining the metabolic state of a cell. To assess this effect, the kinetics of ADK in the absence and presence of ribosomes was measured. The assay measures the generation of ADP from ATP and AMP by using a coupled pyruvate kinase/lactate dehydrogenase reaction. The reaction reduces stoichiometric amounts of NADH that are measured by the native fluorescence of this form of the nucleotide.

In the absence of ribosomes V_{max} increases rapidly reaching a maximum at ~ 1 mM before decreasing at higher ATP concentrations (Figure 3A). The kinetic profile is characteristic of uncompetitive substrate inhibition and suggests the existence of additional ATP binding sites.^{36,37} Uncompetitive inhibition occurs when the inhibitor, in this case one of the substrates ATP, binds to the enzyme–substrate complex to prevent product formation. In the absence of ADK, adding ribosomes did not change the fluorescence of NADH in the presence of ATP. Adding 1 μM ribosome to the enzymatic reaction resulted in a 50% decrease in V_{max} , a 30% decrease in K_{M} , and a 6-fold increase in the inhibition constant, K_{I} (Table 1).

K_{I} was modeled as a second binding site for ATP.^{36,37} Because the ribosome binds to ADK on the side opposite to the ADK active sites (Figure 1C), regulation of ADK activity by the ribosome is likely allosteric. In the context of the model, the results suggest that ADK–ribosome interactions restrict occupancy of the ATP sites required for substrate inhibition. Thus, the consequence of the ADK–ribosome interaction is to maintain ADK in a quinary structural state that limits the maximum velocity of the enzymatic reaction and suppresses the regulatory mechanism of substrate inhibition.

DHFR Binds to Ribosomes

It was suggested that ribosome binding to metabolic proteins, such as DHFR, and TS may be a common phenomenon.^{5,38} Indeed, the in-cell ^1H – ^{15}N CRINEPT-HMQC-TROSY spectrum of [$U\text{-}^2\text{H}$, ^{15}N]–DHFR is broadened suggesting that the protein engages in quinary interactions (Figure S3). To determine if ribosome-specific interactions are present, the NMR spectrum of [$U\text{-}^2\text{H}$, ^{15}N]–DHFR was acquired *in vitro* in the absence and presence of ribosomes (Figure S3). As with ADK–ribosome interactions, the spectra are characteristic of

intermediate exchange between DHFR and ribosomes implying a micromolar binding affinity for the interaction. The broadening of the ribosome spectrum was consistent with DHFR–ribosome quinary interactions.

NADPH Binds to Ribosomes

The binding of DHFR coenzyme NADPH to the ribosome was investigated by using *in vitro* 2D ^1H – ^{31}P -correlation NMR experiments. The change in chemical shifts as the concentration of NADPH is increased was used to quantify bound and unbound species. The dissociation constant resolved for NADPH binding to the ribosome was $4.5 \pm 1.5 \mu\text{M}$, comparable to the dissociation constant resolved for ATP binding to ribosomes (Figure 2C). Unlike ATP, NADPH does not appear to interact nonspecifically with the ribosome at concentrations greater than $\sim 10 \mu\text{M}$.

Protein–Ribosome Interactions Suppress DHFR and Enhance TS Activities

To assess the effect of DHFR and NADPH binding to the ribosome on enzymatic activity, the kinetics of DHFR in the absence and presence of ribosomes was determined. The DHFR assay spectroscopically measures the amount of NADPH consumed to generate tetrahydrofolate, THF, from dihydrofolate, DHF.³⁹ In the absence of ribosomes, DHFR displayed classic Michaelis–Menten kinetics (Figure 3B). Adding $0.5 \mu\text{M}$ ribosome resulted in an $\sim 20\%$ decrease in V_{max} and a 10-fold increase in K_{M} (Table 1) suggesting that the ribosome is a competitive inhibitor of DHFR. The reduction in activity is likely a result of the DHFR–ribosome interface blocking or altering DHF and/or NADPH binding sites, and/or the binding of NADPH to ribosomes which lowers the concentration of free NADPH available for DHFR catalysis.

DHFR and TS are functionally linked in the thymidylate synthetic pathway (Figure 3C). TS uses deoxyuridylate, dUMP, which is mutagenic,⁴⁰ and methylene-THF, Me-THF, to produce deoxythymidylate, dTMP, for DNA synthesis. Ribosome-mediated quinary interactions suppress DHFR conversion of DHF into THF, which is further modified into Me-THF. The decrease in Me-THF decreases TS utilization of the substrate and results in an increase in dUMP. Because TS binds to total RNA (Figure S4) as well as to mRNA⁴¹ and ribosomes,⁵ we examined the possibility that ribosome-mediated quinary interactions also affect TS activity to reduce cellular levels of mutagenic dUMP.

The TS assay measures the oxidation of Me-THF to DHF.³⁰ Adding $0.5 \mu\text{M}$ ribosome resulted in an ~ 20 -fold increase in both V_{max} and K_{M} and a kinetic profile that is characteristic of uncompetitive substrate inhibition³⁷ with a K_{I} of $3.9 \mu\text{M}$ (Table 1 and Figure 3D). The enhancement in TS activity was dependent on the ribosome concentration (Figure 3E). The high affinity resolved for the second binding site for dUMP, K_{I} , is a result of TS–ribosome quinary interactions that both increase the catalytic rate of TS and promote substrate inhibition.

To show the specificity of ribosome-binding interactions on DHFR and TS activities, the activity of another RNA binding protein, Trx,¹⁹ which does not interact with ribosomes, was tested (Figure S5). The Trx activity assay measures the degree of insulin precipitation resulting from reduction by Trx.²⁹ As expected, adding ribosome did not change the time

course of the reaction (Figure 3F). The results are consistent with the observation that the DHFR–ribosome and TS–ribosome interactions are required for the reduction of DHFR and enhancement of TS activities.

GFP Binds to Ribosomes

The high concentration of ribosomes occupies a large fraction of the cytoplasmic volume and contributes to molecular crowding. Molecular crowding affects physical properties such as protein diffusion rates, which are important for growth and adjusting to conditions of stress,^{42–44} and affects folding,^{17,45} assembly,^{46,47} and binding⁴⁸ processes. Previous measurements of GFP diffusion rates in *E. coli* were far smaller than those predicted by scaled-particle theory,⁴⁹ SPT, suggesting that additional factors including macromolecular interactions could be responsible for this observation.^{42,50}

Studies using GFP and GFP-tagged proteins in live cells revealed that diffusion depends on the cell growth rate.⁴² Because the growth rate is linearly related to the ribosome concentration,^{51,52} we postulated that GFP–ribosome interactions might explain the reduction in expected diffusion rates. Adding ribosomes to a GFP variant, emerald GFP or EmGFP, *in vitro* resulted in the almost complete disappearance of the ¹⁵N-HSQC NMR spectrum of [*U*-¹⁵N] EmGFP (Figure S6), suggesting that GFP strongly interacts with ribosomes.

GFP–Ribosome Interactions Slow GFP Diffusion

To examine whether GFP–ribosome interactions affect translational diffusion rates, fluorescence recovery after photo-bleaching, FRAP, was used to quantify the effect of ribosomes on GFP diffusion *in vitro* (Figure 4). SPT, which treats the diffusing species and the molecular crowders as hard-sphere monomers,^{32,49} was used to estimate the ratio of GFP diffusion coefficients in the presence and absence of biopolymer crowding, D/D_0 , as a function of the volume fraction of biopolymer crowders, ν_p . The model has one adjustable parameter, the ratio of the characteristic diffusion length, λ , to the radius of the diffusing protein, R .^{32,49}

Results indicate that ribosomes slow GFP diffusion much more strongly than that expected from simple macromolecular crowding. The fluorescence intensity curves show a decrease in recovery time for GFP in the presence of ribosomes (Figure 4B). GFP diffusion is decreased 2-fold at $\nu_p = 0.06$, which corresponds to 40 μ M ribosome (Figure 4C). By comparison, in-cell where $\nu_p = 0.16$, GFP diffusion was 6 times slower than that observed *in vitro*.⁴² We conclude that the GFP–ribosome interaction is at least partially responsible for the in-cell decrease in GFP diffusion.

This is an important observation since GFP is widely used in cell biology as an innocuous fluorescent tag⁵³ and implies that results obtained with GFP tagged proteins should be re-evaluated to take into account the interaction between ribosome and GFP. Note also that, closely related to translational diffusion,⁵⁴ rotational diffusion plays a major role in the ability to observe proteins by in-cell NMR spectroscopy.⁵⁵ In-cell NMR studies of [*U*-¹⁵N] GFP in *E. coli* revealed that the protein resonances are too broad to be observed by using conventional ¹H–¹⁵N HSQC detection, most likely due to a decrease in the rotational

diffusion rate.⁵⁰ The increased difficulty in observing prokaryotic^{50,56} as compared to eukaryotic in-cell NMR spectra^{15,57–60} likely stems from the fact that the ribosome concentration in eukaryotic cells is on average 10 times lower.⁹

DISCUSSION

Small changes in enzyme catalytic rates can result in large *in vivo* biological effects and lead, for example, to dramatically enhanced antibiotic resistance in the bacterial population.⁶¹ Even though *in vitro* and *in vivo* enzyme catalytic rates generally concur,²² global analyses of *in vivo* catalytic rates in *E. coli* showed on average a 3.5-fold difference from the catalytic rates measured *in vitro*, with some of the rates being suppressed and others enhanced due to unspecified “*vivo-vitro*” effects.²² Proteomics studies have identified a large number of enzymes, including ADK, DHFR, and TS, which are associated with ribosomes or mRNA.^{3–5,38,41,62,63} We showed that the ribosome binds to ADK and DHFR and suppresses the enzymatic activities. The primary coenzymes, ATP and NADPH, which are used in many metabolic pathways, also bind to the ribosome.

For TS, we showed that adding ribosomes *in vitro* enhanced the enzymatic activity ~20-fold, consistent with the ~10-fold increase observed *in vivo*.²² DHFR and TS function together in deoxythymidylate synthesis, and the expression levels of these enzymes have been linked in human cells.⁶⁴ We proposed that ribosome-mediated enhancement of TS activity balances the decrease in the DHFR activity to maintain a low cellular concentration of mutagenic dUMP (Figure 3C). Thus, the ribosome-specific quinary interactions described in this work may have pronounced effects on cellular biochemistry that contribute to the observed differences between enzyme catalytic rates measured *in vivo* and *in vitro*.²²

The micromolar binding affinities of the enzymes and coenzymes for the ribosome suggest that these interactions will take place inside live cells where the ribosome concentration can reach up to tens of micromolars in prokaryotes.⁹ In actively growing *E. coli*, the fractional volume occupied by 70S ribosomes is $V_{S70}/V_{\text{tot}} = 0.16$,⁹ and this ratio may increase by up to four times, ~0.64, outside the space occupied by the nucleoid.^{65–67} Comparing V_{S70}/V_{tot} to the fraction of space occupied by closely packed hard spheres, 0.74,⁶⁸ reveals that *E. coli* ribosomes are tightly packed in the cytosol with “free” space delimited by ribosome surfaces (Figure 5).⁶⁹ These excluded volume effects⁷⁰ increase the effective concentrations of the interacting species by restricting the volume in which biological reactions can take place to the “free” spaces surrounding the ribosomes (Figure 5). This, in combination with attractive chemical interactions,^{71–73} result in large populations of bound species with altered biological activity.

We propose that the ribosome plays a role in organizing metabolism⁷⁴ by serving as a hub where both enzymes and metabolites are concentrated on ribosomal surfaces. In addition to the likelihood of ribosome-specific interactions affecting the cellular biochemistry of individual macromolecules such as metabolic enzymes, the fact that the ribosome concentration is linearly related to cell growth rate^{51,52} adds another, global, layer of biological regulation to growing cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was supported by NIH grant R01GM085006 to A.S.

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Figure 1.

ADK binds to ribosomes *in vitro* and in-cell. (A) (Center) Overlaid *in vitro* ^1H - ^{15}N CRINEPT-HMQC-TROSY spectra of 10 μM [U - ^2D , ^{15}N] ADK without (black) and with 2.5 μM ribosome (red). Surrounding panels show overlays of individual residues including in-cell NMR peaks (blue). (B) Changes in the intensities of ADK peaks due to ribosome binding. Asterisks indicate residues that broaden the most both *in vitro* (above threshold line) and in-cell. Broadening of the peaks in *in vitro* ribosome and in-cell spectra suggest an interaction with the ribosome. I and I_0 are ADK peak intensities with and without the ribosome, respectively. (C) Residues that comprise the ADK-ribosome interaction surface

(red) mapped onto ADK (PDB entry 4AKE³³). These surfaces do not overlap with the substrate, ATP and AMP, binding domains. (D) Fluorescence titration of 0.5 μM ribosome with increasing concentration of ADK. Tryptophan fluorescence was measured at the emission wavelength of 350 nm by using an excitation wavelength of 280 nm. Curve fitting to a single site-binding isotherm resulted in a K_d of $3.7 \pm 0.4 \mu\text{M}$. F_0 is the fluorescence without ADK, and F_{max} is the maximum fluorescence of the ADK–ribosome complex. The fluorescence experiments were performed in triplicate.

Figure 2. Enzyme substrates bind to ribosomes. (A) ATP analogue AMP-PCP binding to ribosomes. The concentration of ribosomes was 2 μM. (B) Overlays of *in vitro* CRINEPT-HMQC-TROSY spectra of 10 μM [*U*-²D,¹⁵N] ADK at 0 μM ATP (blue), 20 μM ATP (magenta), 40 μM ATP (black), and 80 μM ATP plus 1 μM ribosome (red). (C) NADPH binding to ribosomes. The concentration of ribosomes was 1 μM.

Figure 3.

Ribosome interactions modulate enzymatic activity. (A) ADK initial velocity versus ATP concentration without (black) and with (red) 1 μM ribosome. (B) DHFR initial velocity versus DHF concentration without (black) and with (red) 0.5 μM ribosome. (C) The ribosome mediates a compensatory effect on the cycling of dUMP into dTMP. In the presence of ribosomes, DHFR activity is decreased, and TS activity is increased, resulting in a lower concentration of mutagenic cellular dUMP. (D) TS initial velocity versus dUMP concentration without (black) and with (red) 0.5 μM ribosome. (E) Increase in TS activity with increasing ribosome concentration. (F) Trx activity does not depend on ribosome

concentration. Solution turbidity due to insulin precipitation was measured in the presence of (1) Trx; (2) Trx and 0.25 μ M ribosome; (3) Trx and 0.50 μ M ribosome; (4) Trx and 1.0 μ M ribosome; and (5) Trx and 1.5 μ M ribosome.

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Figure 4.

GFP-ribosome interactions slow GFP diffusion. (A) Selected confocal images of 20 μ M EmGFP with and without 40 μ M ribosome 79 ms (top), 236 ms (middle), and 9197 ms (bottom) after photobleaching. (B) Average intensity of the imaging area over time without (■) and with (red circle) ribosome. (C) Upper (solid) and lower (dashed) lines are calculated based on the SPT model with r/R ratios of 0.4 and 0.6, respectively, the range of values measured for globular proteins of the size of GFP[26]. The experimental value for EmGFP diffusion (■) is compared with the *in vivo* value cited in ref 42 (●).

Figure 5.

Ribosomes organize proteins in the cytosol. (Left) Ribosomes are densely packed and limit ADK molecules to the inter-ribosome spaces. Figure adapted with permission from ref 69. Copyright 2006 Elsevier, Inc. (Right) Ribosomes also act as molecular sponges, trapping and releasing cofactors and substrates as the cellular environment changes.

Table 1
Kinetic Parameters Resolved for ADK, DHFR, and TS in the Absence and Presence of Ribosomes

enzyme	[ribosome] (μM)	$V_{\max}(\text{S}^{-1})$	$V_{\max}^{\text{Ribosome}}/V_{\max}^0$	$K_M(\mu\text{M})^b$	$K_i(\text{mM})^b$	R^2
ADK	0	$(4.2 \pm 0.2) \times 10^{-2}$	~0.5	180 ± 20	6.1 ± 0.8	0.98
	1.0	$(2.1 \pm 0.1) \times 10^{-2}$		130 ± 30	35 ± 2	0.96
DHFR	0	$(1.48 \pm 0.04) \times 10^{-4}$	~0.8	0.32 ± 0.04		0.95
	0.5	$(1.16 \pm 0.02) \times 10^{-4}$		3.5 ± 0.1		0.99
TS	0	$(9.7 \pm 0.4) \times 10^{-5}$	~20	5.4 ± 0.7		0.97
	0.5	$(2.0 \pm 0.2) \times 10^{-3}$		120 ± 20	$(3.9 \pm 0.5) \times 10^{-3}$	0.99

^a $V_{\max}^{\text{Ribosome}}$ and $V_{\max}^0$ are the maximum initial velocities with and without the ribosome.

^b Enzymatic parameters in the absence of ribosomes are consistent with those found at <http://www.brenda-enzymes.org>. The sources of the uncertainties are due to both random errors and curve fitting to a model.