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Effects of Recombinant Protein Expression on Green Fluorescent Protein Diffusion in *Escherichia coli*†

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Abstract

Fluorescence recovery after photobleaching was used to measure the diffusion coefficient of green fluorescent protein (GFP, 27 kDa) in *Escherichia coli* in the presence or absence of four coexpressed proteins: cytoplasmic maltose binding protein (42 kDa), tau-40 (45 kDa), α-synuclein (14 kDa), or calmodulin (17 kDa). The GFP diffusion coefficient remains constant regardless of the type of coexpresseed protein and whether or not the coexpressed protein was induced. We conclude that expression of these soluble proteins has little to no effect on the diffusion of GFP. These results have implications for the utility of in-cell nuclear magnetic resonance spectroscopy.

The interior of cells consists of a heterogeneous mixture of macromolecules that are tens to hundreds of times more concentrated than the dilute conditions used for most biophysical studies (1). Such crowding affects the thermodynamic activities of molecules and alters protein chemistry (2). Both theoretical and experimental evidence indicates that the crowded intracellular environment influences a range of biological processes and protein properties including enzyme kinetics, protein folding and aggregation, diffusion, and cell signaling; yet the precise manner by which crowding affects these properties is controversial and not well understood (3–9). Consequently, there is a need to develop analytical techniques, like in-cell nuclear magnetic resonance (NMR)[1] spectroscopy, to probe the interior of living cells.

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Supporting Information **Available:** Additional details on experimental procedures involving plas mid design and modification, protein purification, and cell fractionation methods (for cellular location studies), as well as SDS–PAG from cell fractionation. This material is available free of charge via the Internet at http://pubs.acs.org.

NMR spectroscopy shows promise as a noninvasive method for monitoring proteins in cells (10). However, obstacles arise because NMR is insensitive. To obtain high-quality spectra, the protein of interest must be expressed at higher concentrations than other cellular components, which raises concerns about the relevancy of the observations from in-cell NMR. One might expect protein production to cause further crowding, yet Dedmon et al. showed that induced and uninduced *Escherichia coli* cells have the same amount of total protein (11). This observation suggests that cells compensate for overexpression by decreasing the level of other components. Methods for increasing protein expression are known, but it is not known how overexpression affects cellular dynamics (12).

Intracellular protein diffusion should provide some insight about the effects of protein overexpression on the cellular environment. In the simplest case, diffusion is expected to be inversely related to the effective viscosity, which in turn depends on the concentration of macromolecules in cells (13). Furthermore, insight about intracellular diffusion is important in its own right because diffusion is crucial for metabolism, gene transcription, protein assembly, macromolecular interactions, signaling, and other regulatory functions (13). For bacteria, Brownian motion often serves as the primary source of intracellular movement, because these cells lack the motor proteins of higher organisms (14).

Fluorescence recovery after photobleaching (FRAP) is a wellestablished technique used for obtaining translational diffusion coefficients (15, 16). In FRAP, a small region of a fluorescent sample is typically bleached with a laser, and fluorescence recovery is monitored as the unbleached fluorescent markers from the surrounding regions diffuse into the bleached area. Although the technique has traditionally been applied to eukaryotic cells, Elowitz and co-workers pioneered FRAP experiments in *E. coli* (14). This work paved the way for diffusion studies involving the effects of osmotic shock and crowding (17, 18). Of specific interest, van den Bogaart et al. reported no correlation between the GFP diffusion coefficient and the fluorescence intensity of *E. coli* cells (which was assumed to reflect GFP expression levels) (19). This result is seemingly inconsistent with the observation by Elowitz et al. that increasing the inducer concentration significantly reduces the diffusion coefficient of GFP (14). Since neither study directly quantified protein concentration, the discrepancy may involve a difference in expression levels.

Here, we use green fluorescent protein (GFP, 27 kDa) as a tracer molecule and measure its intracellular diffusion in the presence and absence of four different proteins expressed at varying levels in *E. coli*. GFP is globular, and the version we use is nondimerizable (20). The four proteins include two globular proteins, maltose binding protein (MBP, 42 kDa) and bovine calmodulin (17 kDa), and two disordered proteins, human tau-40 (45 kDa) and human α-synuclein (14 kDa). MBP is normally found in the periplasm, but we use a version that is expressed in the cytoplasm (21). Calmodulin is a calcium binding protein found in eukaryotes that regulates numerous enzymes (22). α-Synuclein is associated with Parkinson's disease (23). When expressed in *E. coli*, this protein has been reported to be

[1]Abbreviations: D_{GFP}, diffusion coefficient of GFP; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; IPTG, isopropyl *β*-D-thiogalactopyranoside; LB_{AMP}, Luria broth containing 100 μg/mL ampicillin; MBP, maltose binding protein; NMR, nuclear magnetic resonance; OD_{600}, optical density at 600 nm; SDS–PAG, sodium dodecyl sulfate–polyacrylamide gel.

found exclusively in the periplasm (24). We, however, find it in both the cytosol and the periplasm (*vide infra*). Tau-40 is a microtubule-associated protein commonly found in neurons. It is highly soluble, but when misfolded, it can form aggregates that contribute to neurodegenerative disorders such as Alzheimer's disease (25). Together, these four model proteins provide a range of size and structure for probing the effect of protein expression on intracellular dynamics.

Experimental Procedures

Protein Expression

Four vectors derived from AcGFP1 (Clontech) and pBAD/HIS C (Invitrogen) were created to contain the gene for a nondimerizable GFP (20) under the *lac* promoter and the gene for either α-synuclein, tau-40, MBP, or calmodulin under the control of the *araBAD* promoter (see Supporting Information). These vectors were individually transformed into competent *E. coli* BL21-AI cells (Invitrogen) and plated on Luria broth plates containing 100 *μg/mL* ampicillin (LB_{AMP}). A 5 mL starter culture of liquid LB_{AMP} was inoculated with a single colony and grown overnight at 37 °C with constant shaking at 225 rpm. This starter culture was used to inoculate 25 mL of fresh LB_{AMP} in a 250 mL flask at a 1:25 dilution. Once the optical density at 600 nm (OD_{600}) reached 0.5–0.7, the culture was divided into three 5 mL aliquots, which were induced with arabinose according to Figure 1 and grown at 37 °C with constant shaking at 225 rpm. After 3 h, the OD_{600} of each sample was measured, and chloramphenicol, to halt expression, was added to a final concentration of 50 *μg*/mL.

Sample Preparation

Cover glasses (22 × 22 × 0.17 mm, no. 1.5; Zefon) and glass microscope slides (3 in. × 1 in. × 1 mm; Fisher Scientific) were boiled in ICN detergent (MP Biomedicals) for 10 min, bath-sonicated for 30 min, rinsed thoroughly with deionized water, and dried overnight at 160 °C. The dried slides were cleaned in an Ar-ion plasma cleaner (PDC-3XG; Harrick Scientific) for 15 min at 25 °C immediately prior to use. The cover glass slides were pretreated with a 0.01% (w/v) poly(L-lysine) solution (Sigma-Aldrich) for 15 min, rinsed with minimal media [7.6 mM $(NH_4)_2SO_4$, 60 mM K_2HPO_4, 2 mM $MgSO_4$, 20 *μM* $FeSO_4$,1 mM EDTA (pH 6.8)], and attached to a microscope slide with double-sided tape (part no. 021200-64988; 3 M Corp.) to form a sandwich. For osmotic stress and urea measurements, the minimal media rinse contained either 250 mM sorbitol (390 mOsm, measured with a Vapro osmometer) or 500 mM urea. The cultures, prepared as described above, were injected into the sandwiches. After incubating for 30 min at 25 °C, the sample chamber was rinsed with minimal media and sealed with vacuum grease.

Determining Protein Concentration

For each of the four cultures, 1 mL aliquots were collected 0 and 2 h after adding chloramphenicol. These aliquots were centrifuged for 10 min at 8000 *g* (Eppendorf model 5418). The pellets were resuspended in 20 mM potassium phosphate buffer (pH 7.5). Protein standards were obtained by using the purification methods described in the Supporting Information. The concentration of each standard was determined by absorbance (GFP, $\varepsilon_{475nm} = 32500\ cm^{-1}\ M^{-1}$ as reported by the manufacturer, Clontech; MBP (26), ε_{280nm} =

69000 cm^{-1} M^{-1}; calmodulin (27), ε_{276nm} = 3030 cm^{-1} M^{-1}) and/or with the Lowry (28) method (modified Lowry protein assay kit; Pierce) using cytochrome *c* (for α-synuclein and calmodulin) or ovalbumin (for tau-40 and MBP) as a reference. The proteins in cell lysates and standards were resolved by electrophoresis on 10-20% gradient sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGs; Criterion, Bio–Rad) for 60 min at 200 V. Gels were analyzed by both fluorescence and Coomassie staining with a VersaDoc MP imager (Bio-Rad). The exposure time was optimized to prevent pixel saturation, and Quantity-One software (Bio-Rad) was used to quantify the band intensities. Calibration curves of the standards were used to determine the amount of the proteins of interest in the cell lysates. The cell densities of the cultures were determined from the OD_{600} (29). These values were confirmed by dilution plating. The intracellular protein concentrations, *C* (in millimolar), shown in Table 1 were calculated by using the equation:

$$C = \left(\frac{n}{NV_{\text{cell}}}\right)\left(\frac{V_p}{V_{\text{load}}}\right)$$

where *n* (in millimoles) is the amount of protein contained in the band on the SDS–PAG (based on the standard curve made from pure proteins), V_p (in microliters) is the volume of resuspended pellet (in phosphate buffer with SDS–PAG loading dye), V_{load} (in microliters) is the volume of resuspended pellet loaded into the SDS–PAG, *N* is the number of cells in the 1 mL aliquot (determined by the OD_{600}) (29), and V_{cell} is the volume of an *E. coli* cell (1×10^{-15} L) (30). Measurements were performed in triplicate.

Microscopy

The intracellular GFP diffusion coefficient was measured by using FRAP (14). Single cell images were recorded with a Zeiss 510 Meta scanning confocal inverted microscope equipped with a 30 mW argon laser. The sample was imaged with a 63×, 1.4 NA plan-apochromat oil-immersion objective and a pinhole of 2.0 Airy-disk units. An excitation wavelength of 488 nm was selected. A 505 nm long-pass filter was used for detection. Cells were oriented in the *x* direction, and the laser bleach spot was moved to one pole of the cell. For photobleaching, the laser intensity was ∼240 μW, with a ∼0.2 μW observation beam. After 99 prebleach images were collected, the region selected for bleaching was scanned for 52 ms, and 500 postbleach images of 128 by 16 pixels (7.3 by 0.9 μm) were recorded at a rate of 13.1 ms/frame.

Data Analysis

Time series of fluorescence images were analyzed as described (18). In short, images were converted to one-dimensional intensity profiles, $I(x,t)$, by averaging columns of pixel intensities (perpendicular to the long cell axis) as a function of distance from the cell edge (x). The one-dimensional profiles were used to calculate Fourier amplitudes $A_n(t)$:

$$A_n(t) = \frac{2}{L}\int_0^L \cos(q_n x) I(x,t)\,\mathrm{d}x$$

where $q_n = n\pi/L$, t is time, L is the cell length in micrometers, and $n = 1$, because the larger numbered n-terms decay too quickly to be measured. The Fourier amplitudes were plotted as a function of time and fit to a single exponential decay, $a[\exp(-bt)] + c$, with a, b, and c as free parameters. Diffusion coefficients (D) were obtained from the equation $b = Dq_1^2$. Analysis was performed using Mathematica (Wolfram).

Results

Intracellular Protein Concentrations

Four vectors were created. The GFP gene was placed under the *lac* promoter. The *araBAD* promoter was used to control the expression of α-synuclein, MBP, tau-40, or calmodulin. Figure 1A shows typical Coomassie-stained gels of the *E. coli* cell lysate. For a given system, GFP is expressed at a constant level as shown by the Coomassie-stained gels (Figure 1A) and as suggested by the fluorescent gels (Figure 1B). The amount of coexpressed protein is controlled by the amount of arabinose used for induction. In the absence of arabinose, GFP is expressed and detected, but the coexpressed protein (MBP, calmodulin, tau, or α-synuclein) is undetectable. The same result is obtained from cell lysates electrophoresed before or 2 h after adding chloramphenicol to stop protein synthesis. This observation shows that the intracellular protein concentration does not change during the FRAP experiments.

SDS–PAG electrophoresis was used to quantify the concentration of a given protein in cells. The results are summarized in Table 1. Comparing the induced and uninduced GFP concentrations shows that the value is the same whether or not another protein is coexpressed. The level of GFP expression varies, however, from system to system. The most extreme variation is a 5-fold increase in GFP expression in the MBP system compared to the α-synuclein system. With the exception of tau-40, the GFP concentration appears to be inversely related to the concentration of the coexpressed protein.

Protein Location

Four fractionation methods were used to determine the locations of the coexpressed proteins: osmotic shock (24, 31), exposure to detergent (32), osmotic shock plus lysozyme (33), or chloroform treatment (34). Coomassie-stained gels (see Supporting Information) of the supernatants (periplasmic proteins) and pellets (cytoplasmic proteins) confirm that MBP and tau-40 are present exclusively in the cytoplasm. The supernatant from the chloroform fractionation contained calmodulin, yet results from the other three methods suggest that calmodulin is exclusively localized to the cytoplasm. All fractionation methods show the presence of α-synuclein in both the cytoplasm and periplasm. The percent of cytosolic α-synuclein ranged from 40% (chloroform) to 70% (lysozyme), depending on the method.

Protein Expression

SDS–PAG electrophoresis of cell lysates provides bulk information about expression. To examine cell-to-cell variation, it is necessary to observe individual cells. The fluorescence intensity is constant from cell to cell in *E. coli* coexpressing GFP and α-synuclein (Figure 2A). Similar images were obtained for the calmodulin and MBP systems (not shown). In

contrast, the images of *E. coli* coexpressing tau-40 (Figure 2B) show bright and dim cells, suggesting that the GFP concentration varies from cell to cell. Inexplicably, this intensity variation for the tau-40 system is observed whether or not the tau-40 expressing cells are induced. For all images, the distribution of GFP within a single cell is uniform within optical resolution, suggesting that GFP is neither aggregated nor localized to specific regions, under the conditions examined here.

The expression of the four proteins listedinTable1isdriven by the *araBAD* promoter. Since the levels of these *coexpressed* proteins cannot be visualized in cells, GFP was expressed under the *araBAD* promoter to assess the cell-to-cell expression level variation for this promoter. The cells in Figure 2C are dimmer than the cells in Figure 2A because the *araBAD* promoter is weaker than the *lac* promoter for GFP. The fluorescence intensity is constant for *E. coli* expressing GFP under *araBAD* at 0.02% arabinose (Figure 2C). Histograms of the intensity versus the number of cells made from the data in Figure 2 indicate a slightly wider spread of fluorescence intensities for GFP under the *araBAD* promoter (Figure 2C) compared to GFP under the *lac* promoter (Figure 2A), but both appear to be normally distributed (data not shown). This result indicates that, above the arabinose saturation concentration, *araBAD*-driven expression of GFP is similar from cell to cell. By inference, this result suggests that the other proteins driven by this promoter should also show constant expression from cell to cell.

Controls for FRAP Experiments

The photochemical properties of fluorophores can sometimes hinder experiments. For instance, GFP variants have been reported to photobleach reversibly and undergo photoinduced cross-linking (35, 36). To assess reversible photobleaching, confocal microscopy was used to bleach the GFP throughout an entire cell and to monitor the intracellular fluorescence over time. Postbleach fluorescence recovery is not observed, indicating that GFP is irreversibly photobleached on the time scale of our experiments. Likewise, when experiments were conducted without a bleach pulse, no fluorescence decay is observed. To ensure that GFP does not undergo bleach-induced photochemistry, FRAP runs were repeated on the same cell several times. The same diffusion coefficient is obtained each time, suggesting that the system is not significantly altered by photochemistry.

Intracellular Diffusion of GFP

FRAP was used to measure the apparent diffusion coefficient of GFP in cells coexpressing an additional recombinant protein. Figure 3A shows one-dimensional pixel intensity profiles of a typical cell as a function of distance from the cell edge at different postbleach times. The observation that the fluorescence is constant with respect to the position at long times implies that either all or most of the GFP has long-range lateral mobility. Figure 3B shows the amplitude of the first Fourier mode ($n = 1$) for the same cell (blue), which is consistent with previous experiments (18). The apparent diffusion coefficient was determined from the decay rate of this amplitude (Experimental Procedures). The average diffusion coefficients measured are summarized in Figure 4. Regardless of the specific protein or the arabinose concentration used to induce expression, the intracellular diffusion coefficient of GFP,

D_{GFP}, is the same within the uncertainty of the measurement. Likewise, the addition of 500 mM urea to the culture did not affect D_{GFP}, suggesting that GFP diffuses as a monomer.

Osmotic Shock

To confirm that our implementation of the FRAP experiment is sensitive enough to detect differences in D_{GFP}, cells were osmotically shocked with sorbitol. A 226 mOsm increase in the osmolarity decreased the decay rate of the first Fourier amplitude 3-fold (Figure 3B). This decrease is consistent with previous studies (18).

Discussion

We expanded upon the simplistic suggestion of Dedmon et al. by examining the effects of protein expression on intracellular diffusion. Dedmon et al. proposed that if intracellular protein concentration is constant, then overexpression of a single protein should not affect protein diffusion (11). Overexpression, however, could alter other factors, such as cytoplasmic composition and protein interactions, both of which could affect protein diffusion. Thus, a more direct measurement of the effects of overexpression on diffusion was necessary.

For a given system, GFP was expressed at a constant level and its diffusion coefficient, D_{GFP}, was measured in the presence and absence of a second recombinant protein. Four proteins were chosen to cover a range of characteristics. Both large (MBP, 42 kDa; tau-40, 45 kDa) and small (α-synuclein, 14 kDa; calmodulin, 17 kDa) proteins were selected because evidence suggests that the size of the crowding agent affects diffusion (37). Globular (MBP and calmodulin) and disordered (α-synuclein and tau-40) proteins were chosen to determine if shape is a factor. When induced with arabinose, the coexpressed protein represented as much as 15% of the mass of the total intracellular protein (9). Despite these variations in protein size, shape, and concentration, D_{GFP} remained within error of the commonly referenced GFP diffusion coefficient in *E. coli*, 7.7 ± 2.5 $\mu m^2 s^{-1}$ (14). D_{GFP} was also consistent with the value recently measured by total internal reflection continuous photobleaching (38). Our observations show that GFP diffusion is independent of the type or amount of protein coexpressed, but several points need to be addressed.

GFP aggregates could complicate the interpretation. Almost all intracellular protein diffusion studies in *E. coli* have involved GFP or GFP-fusion proteins. GFP may interact with other proteins inside the cell, which would decrease D_{GFP}. To test these potential protein-protein interactions, 500 mM urea was added to the cells. *E. coli* take up urea and remain viable at these concentrations (39, 40). The addition of urea did not alter the observed diffusion coefficient, implying that GFP is not interacting with itself or intracellular components.

FRAP could be too insensitive to changes in diffusion. Osmotic shock by sorbitol increases the intracellular concentration of macromolecules, which significantly decreases protein mobility (41). To show that our instrumentation is sufficiently sensitive to detect differences in D_{GFP}, the GFP diffusion coefficient was measured in cells treated with 250 mM sorbitol. Dilution plating confirmed that sorbitol does not affect cell viability. We obtained a D_{GFP} of

2.4 ± 1.6 $\mu m^2 s^{-1}$ in 397 mOsm buffer, which is consistent with other studies that report diffusion coefficients of 1.8 $\mu m^2 s^{-1}$ in 370 mOsm buffer (19) and 0.94 ± 0.55 $\mu m^2 s^{-1}$ in 392 mOsm buffer (18).

The issue of whether protein expression is uniform across the population of cells must be addressed. Expression from the *araBAD* promoter is regulated by arabinose, such that intermediate levels of expression can be achieved by using subsaturating arabinose concentrations. The intracellular concentrations of the coexpressed proteins (Table 1) were determined from gels like the one shown in Figure 1 and represent average intracellular concentrations. It is known, however, that there is cell-to-cell variability of expression levels at subsaturating inducer concentrations. Siegele and Hu (42) showed that intermediate levels of bulk expression result from a mixture of fully induced and uninduced cells. Thus, increasing the arabinose concentration increases the fraction of induced cells, rather than the amount of protein produced in an individual cell. Their results show that 0.02% arabinose is above the saturation level such that most of the cells are fully induced. One explanation for the few cells that were not induced is that they lost the vector, containing the *araBAD* promoter. Our images of *E. coli* expressing GFP under the *araBAD* promoter induced with 0.02% arabinose verify this observation (Figure 2C). Similar images of cells expressing GFP under the *lac* promoter show that GFP expression is uniform from cell to cell, with the exception of tau-40 (Figure 2A,B).

Due to the behavior of the *araBAD* promoter at subsaturation, the diffusion coefficients in Figure 4 and the intracellular concentrations in Table 1 are provided for only fully induced (≥0.02% arabinose) and uninduced cultures. Nevertheless, intermediate levels of all coexpressed proteins yield GFP diffusion coefficients within error of those given in Figure 4 (not shown).

Interpreting the effect of the protein coexpression on D_{GFP} depends on the assumption that the protein of interest is in the same compartment as GFP. It is possible that no effect on GFP diffusion by the expression of a second protein arises because the protein was not expressed in the same compartment as the GFP. An *E. coli* cell comprises two main compartments: the periplasm and the cytoplasm. The periplasm is the outer compartment bounded by the plasma membrane and cell wall. It encloses 20–40% of the cell's total volume (43). The cytoplasm comprises the volume surrounded by the plasma membrane. The cytosol is the cytoplasm without other subcellular structures, such as ribosomes, and the fibrous proteins that determine cell shape, motility, and material transport. GFP is found exclusively in the cytosol of *E. coli* (44).

To determine if the coexpressed proteins are present in the cytoplasm or periplasm, the cells were subjected to osmotic shock (24, 31), detergent (32), osmotic shock plus lysozyme (33), or chloroform (34). SDS–PAG electrophoresis of the supernatants (periplasmic proteins) and pellets (cytoplasmic proteins) confirmed that the coexpressed proteins are soluble and present in the cytoplasm. The chloroform fraction of calmodulin was inconsistent with the results from the other methods. However, even for MBP, α-synuclein, and tau-40, the chloroform supernatant contains numerous proteins that are absent from the supernatants from the other fractionation methods. This observation suggests that chloroform can lyse

entire cells and not just the periplasm. On the basis of the osmotic shock, lysozyme, and detergent experiments, we conclude that calmodulinis a cytosolic protein. Our observation that 40–70% of the α-synuclein is cytosolic is inconsistent with a study that reports α-synuclein as exclusively periplasmic in *E. coli* (24). Another study, which shows 60–85% of α-synuclein in the periplasm, suggests that the amount depends on the strain used for expression (34). The BL21-AI strain used here was not part of either previous study. This difference in *E. coli* strain may explain the inconsistency in the amount of periplasmic α-synuclein. In summary, all of the proteins except α-synuclein are found exclusively in the cytosol.

The tau-40 expression system was unlike the others. As shown in Table 1, tau-40 expression is 3-fold lower than the other proteins when the same amount of inducer is added. Furthermore, GFP expression with this system exhibits extensive cell-to-cell variation (Figure 2B). This observation suggests that arabinose saturation has not been reached at 0.02%. The results with 0.2% arabinose are the same as those from a 0.02% sample, and 2% arabinose resulted in even lower expression. Nevertheless, the GFP diffusion coefficients for both induced and uninduced cultures were consistent with the other systems.

These control experiments strengthen our general conclusion that expression of these proteins has little or no effect on the diffusion of GFP. This diffusional homeostasis softens one criticism of in-cell NMR, namely, that the protein overexpression required for observing in-cell spectra leads to an unacceptably nonphysiological environment. Therefore, the need to over-express a protein for in-cell NMR does not necessarily invalidate the physiological relevancy of the results. The conclusion also raises new questions. It will be important to know what maintains diffusional homeostasis in the face of a single protein representing >10% of a cell's protein. In addition, the effects of inclusion bodies (which occur when overexpression leads to insolubility) need to be understood.

Our conclusion also highlights an enigma. NMR spectra from several soluble globular proteins cannot be observed in intact *E. coli* cells (10). Yet, inclusion bodies are not observed, the soluble protein is easily purified, and simply lysing the cells causes the appearance of high-quality spectra. One explanation is that the high viscosity in cells slows the protein's rotation, making its resonances too broad to observe (45).

The data presented here show that translational diffusion in cells is slowed about 10-fold compared to dilute solution. The enigma arises in trying to reconcile this 10-fold decrease with *in vitro* data. Adding enough of the macromolecular crowder, 40 kDa polyvinylpyrrolidone, to slow translation of a small globular protein 10-fold slows rotational diffusion by only 2-fold (10). A 2-fold decrease in rotational diffusion is not enough to obliterate the spectrum of a small globular protein. It remains unclear how the cellular interior retards rotational diffusion to such a degree that protein NMR spectra cannot be observed, while having such a small effect on translational diffusion.

In summary, the data presented here indicate that protein coexpression has little to no effect on the intracellular diffusion of GFP. These observations suggest that in-cell NMR can provide biologically relevant data, despite the need to overexpress the protein being studied.

However, the results also highlight new questions that must be addressed about cellular protein dynamics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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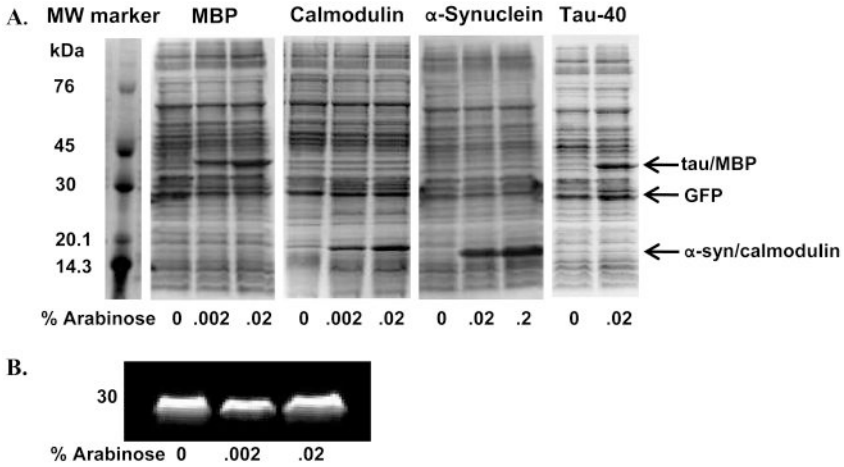

Figure 1.

Intracellular GFP concentration is independent of the amount of an individual coexpressed protein. (A) Cell lysates were separated on a 10–20% gradient SDS–PAG and visualized with Coomassie staining. The arabinose concentrations were adjusted for each system to maximize protein expression with the least amount of inducer. (B) GFP can be visualized by using fluorescence, as shown by these lysates from cells coexpressing GFP and MBP. Similar results were observed for the other three systems.

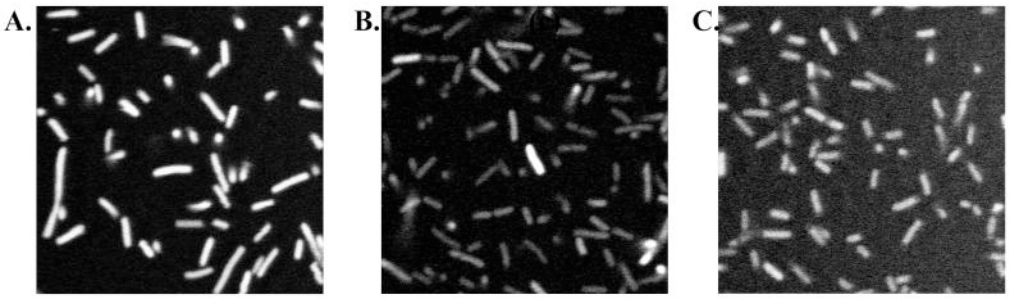

Figure 2.

Fluorescence images (35 μm × 35 μm) of *E. coli* expressing GFP. In panels A and B, GFP, under the *lac* promoter, is coexpressed with (A) α-synuclein or (B) tau-40, both under the *araBAD* promoter. In panel C, GFP is expressed under the *araBAD* promoter, instead of the *lac* promoter, with no coexpressed protein.

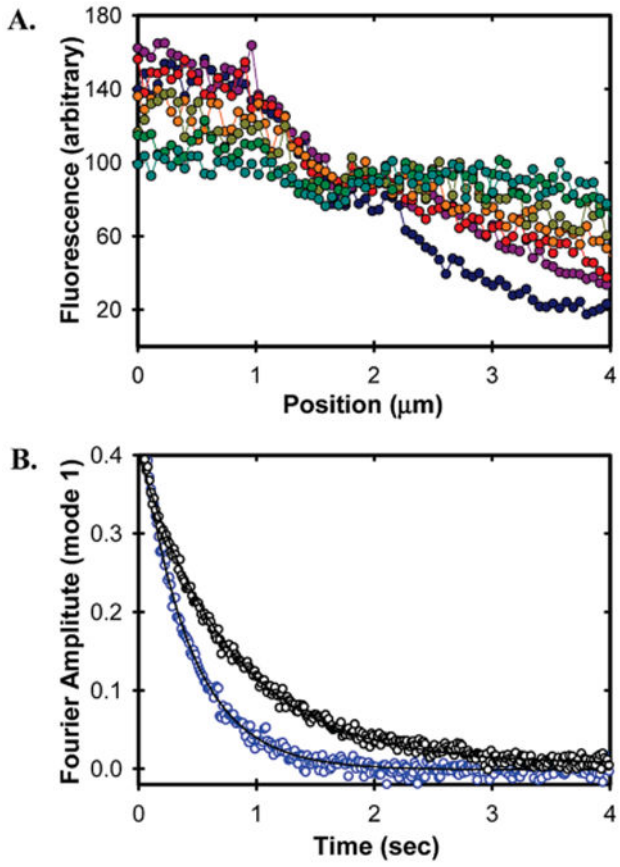

Figure 3.

Photobleaching data. (A) Fluorescence intensity profiles at 0.03 (dark blue), 0.10 (purple), 0.17 (red), 0.30 (orange), 0.50 (yellow), 0.83 (green), and 1.51 (cyan) s after photobleaching are shown for a BL21-AI cell coexpressing tau-40 and GFP. (B) Temporal decay of the first Fourier amplitude for the same cell (blue) and for a cell exposed to a 226 mOsm increase in osmolality (black). The solid lines are fits to the exponential function, $a[\exp(-bt)] + c$, with a, b, and c as free parameters.

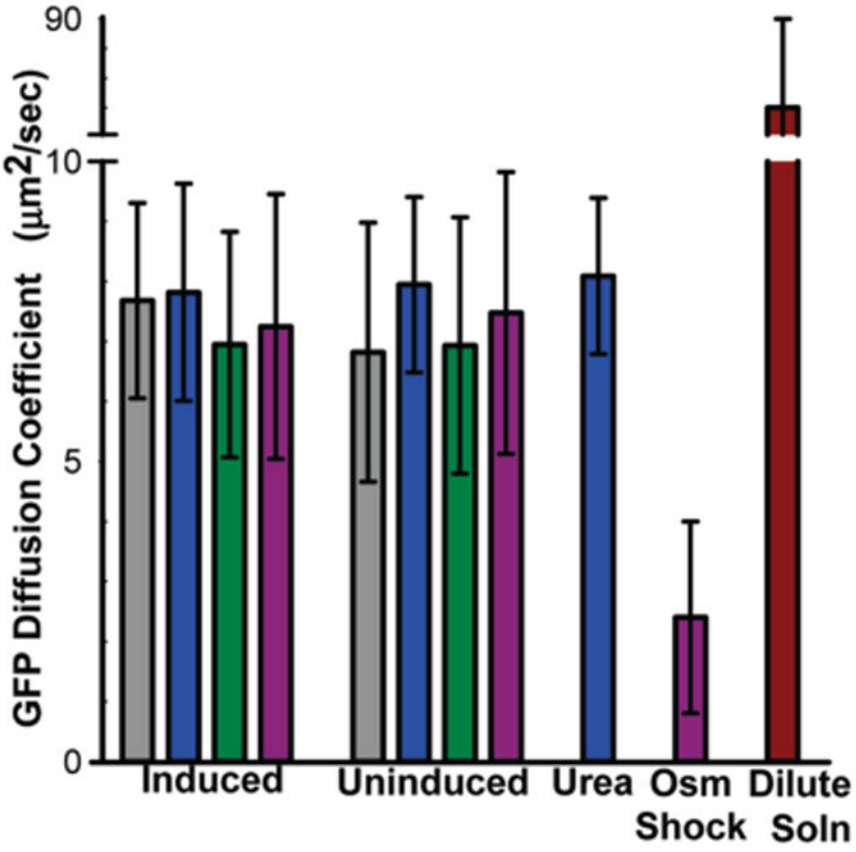

Figure 4.

Coexpressing another recombinant protein does not affect intracellular GFP diffusion. GFP was coexpressed with either calmodulin (gray), MBP (blue), tau-40 (green), or α-synuclein (purple). The arabinose concentrations used for induction were 0.02%, 0.02%, 0.02%, and 0.2%, respectively. GFP diffusion in the absence of inducer (arabinose) from these same coexpression systems is also shown ($n > 34$). GFP was expressed constitutively from an uninduced *lac* promoter. The induced MBP sample was exposed to 500 mM urea ($n = 21$). Osmotic shock was introduced by adding sorbitol to a final concentration of 250 mM ($n = 19$) to the α-synuclein sample. For comparison, the published (46) GFP diffusion coefficient in dilute solution (4.5 μM) is also shown.

Table 1

Intracellular Concentrations[a]

	coexpressed protein	GFP	
	induced (mM)	uninduced (mM)	induced (mM)
MBP	0.6 ± 0.1[b]	0.9 ± 0.5	1.0 ± 0.2
calmodulin	0.8 ± 0.3	0.6 ± 0.3	0.6 ± 0.3
tau-40	0.21 ± 0.06	0.5 ± 0.1	0.5 ± 0.2
α-synuclein[c]	1.27 ± 0.09	0.16 ± 0.06	0.19 ± 0.07

[a] Quantified by integrating pixel intensities of bands from Coomassie-stained SDS–PAGs.

[b] Standard deviation, $n = 3$.

[c] Some of this protein is periplasmic (see text).