Single-molecule investigations of the stringent response machinery in living bacterial cells

Brian P. English[a], Vasili Hauryliuk[a,b,1], Arash Sanamrad[a,1], Stoyan Tankov[a,b], Nynke H. Dekker[a,c], and Johan Elf[a,d,2]

[a]Department of Cell and Molecular Biology, Uppsala University, Uppsala, Sweden; [b]University of Tartu, Institute of Technology, Tartu, Estonia; [c]Department of Bionanoscience, Kavli Institute of Nanoscience, Delft University of Technology, Delft, The Netherlands; and [d]Science for Life Laboratory, Uppsala University, Uppsala, Sweden

AUTHOR SUMMARY

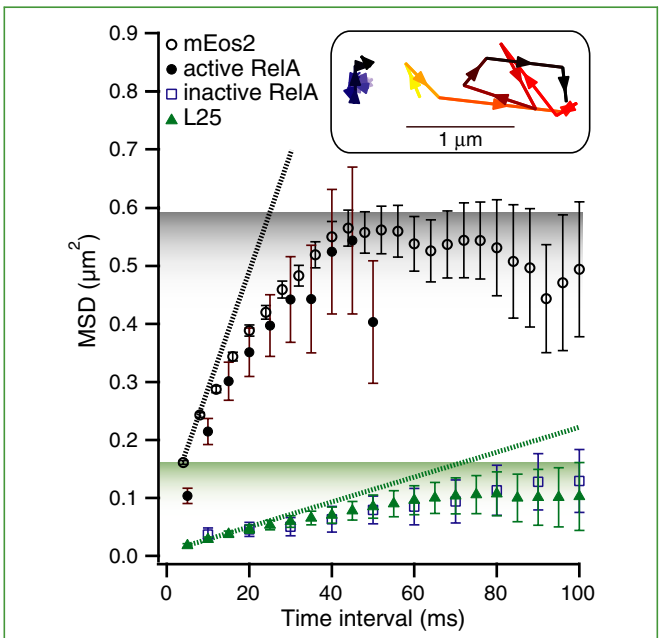

Fig. P1. Diffusion comparisons. Mean square displacements (MSDs) for mEos2 (in black), ribosomal protein L25 (in green), active RelA (in brown, with 2.5 mM of L-Serine Hydroxamate), and for inactive RelA diffusion (in blue, in exponentially growing cells) with error bars representing standard errors of the means. Inactive RelA diffusion is indistinguishable from the diffusion of Dendra2-labeled ribosomes. When cells are starved, the diffusion of RelA changes dramatically and is similar to that of mEos2. (*Inset*) When the temperature is held constant, only slowly diffusing RelA trajectories are observed (left trajectory, recorded with a frame time of 10 ms). During the temperature jump from 21 °C to 37 °C, one transiently observes fast diffusion trajectories of RelA (right trajectory, with a frame time of 10 ms).

Enzymes confer speed and specificity to chemical reactions in living cells. The properties of enzymes are commonly investigated using purified components in test tubes. But how enzymes operate in their natural environment inside the living cell remains an important question in enzymology. Here we combine biophysical and imaging techniques to create a method by which individual enzyme molecules can be studied in live bacterial cells with submicrometer spatial precision and millisecond temporal resolution. The object of our investigation is the *Escherichia coli* enzyme RelA, which plays a key role in a stress adaptation mechanism called the stringent response. During the stringent response, the small messenger molecule ppGpp is made by RelA in response to different kinds of stress. ppGpp binds and modulates components involved in important cellular processes, including transcription, translation, and replication. The catalytic mechanism of RelA is not known in detail but is tightly linked to the enzyme's association to and dissociation from the cell's protein-synthesizing machinery, the ribosome. Binding to the ribosome dramatically changes the diffusion properties of the individual enzyme molecule. We use this change as a "readout" of the RelA catalytic cycle. We show that in unstressed cells, RelA is tightly associated with ribosomes. When cells are permanently stressed, RelA freely diffuses in the cytoplasm, which, in conjunction with its documented enzymatic activity under these conditions, suggests that the enzyme in its catalytically active state is dissociated from the ribosome. When stressed transiently by heat shock, RelA temporarily dissociates from the ribosome and rebinds upon cellular adaptation. These observations helped formulate a model for the RelA catalytic mechanism, an "extended hopping model."

Cells use enzymes as tools for the accumulation and storage of energy, production of cellular building blocks, sensing of the extracellular environment, and regulation of complex cellular metabolism. Our current understanding of how enzymes work is derived from the study of enzymes that are extracted from cells and purified to a state of apparent homogeneity. These proteins are then almost invariably studied in bulk by kinetic methods that follow the averaged progression of enzymatic reactions of billions of individual molecules.

In this in vitro approach, the validity of the model system could be called into question: Can the regulatory process be fully captured by studying isolated enzymes in nonnative environments? This is a relevant concern for the study of key regulatory enzymes that act as environmental sensors. First, test-tube reactions typically include only a small subset of cellular components, and some interaction partners may be unknown. Secondly, it might be impossible to recreate the appropriate proportions of all potential binding partners in vitro. An alternative is an in vivo investigation of the interaction kinetics. The in vivo approach has its own set of challenges related to studying a mix of unsynchronized binding and dissociation reactions: Some kinetic approaches, such as ligand chase ensemble experiments, are infeasible in living cells. Therefore, a direct investigation of the enzymatic cycle

Author contributions: B.P.E. and J.E. designed research; B.P.E., V.H., A.S., S.T., N.H.D. and J.E. performed research; B.P.E., V.H., A.S., and N.H.D. analyzed data; B.P.E. performed ribosome, mEos2, and RelA cloning, tracking and analysis, and bulk growth experiments; V.H. performed mEos2 and RelA tracking and analysis and bulk growth experiments; A.S. performed mEos2 tracking and analysis, algorithm development, and bulk growth experiments; S.T. performed mEos2 tracking and analysis and bulk growth experiments; N.H.D. performed RelA cloning and tracking and bulk growth experiments; and B.P.E., V.H., A.S., N.H.D., and J.E. wrote the paper.

The authors declare no conflict of interest.

This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

[1]V.H. and A.S. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: johan.elf@icm.uu.se.

See full research article on page E365 of www.pnas.org.

Cite this Author Summary as: PNAS 10.1073/pnas.1102255108.

requires experiments that are performed inside living cells and on the single molecule level.

The bacterial enzyme RelA is a central metabolic regulator involved in sensing and responding to stress by producing the small messenger molecule ppGpp (1). We create an experimental approach by combining single molecule tracking under stroboscopic illumination (2) and photoconversion (3). Stroboscopic illumination allows for reliable detection of fast and freely diffusing molecules in the bacterial cytoplasm. The use of a photoconvertible variant of the green fluorescent protein (GFP) Dendra2 as a label allows for detection of individual target molecules irrespective of their cellular copy numbers. We have labeled RelA with Dendra2 using an approach that allows us to follow the diffusion of single RelA molecules expressed at physiological copy numbers in live *E. coli* cells with submicrometer spatial precision and millisecond temporal resolution.

Previous in vitro biochemical investigations suggested that RelA binding to and dissociation from the ribosome are key events in its catalytic cycle (4). These interactions are expected to change the diffusion properties of RelA markedly, allowing us to obtain readouts of its catalytic state directly from the diffusion of individual RelA molecules. As a reference for the diffusion of a small cytosolic protein, we tracked the GFP variant mEos2, and as a reference for ribosome-associated RelA we tracked ribosomes labeled by fusing the chromosomal gene encoding the ribosomal protein L25 with the gene encoding Dendra2. These two references have markedly different diffusion characteristics; mEos2 is fast and diffuses freely throughout the volume of the bacterial cell (Fig. P1, in black). Ribosomes, on the other hand, are much slower and confined, being spatially restricted in their diffusion (5). These pronounced differences allow the discrimination between free and ribosome-bound RelA.

We studied RelA under two types of stress that are mediated by the enzyme: amino acid starvation caused by the addition of the amino acid analogue L-serine hydroxamate (SHX), and heat shock. Our data shows that in unstressed cells, RelA is tightly associated with ribosomes (Fig. P1, in blue), and when activated, it dissociates from the complex for a prolonged duration. Under permanent stress caused by SHX, RelA is observed to come off the ribosome (Fig. P1, in brown), suggesting that it is catalytically active while freely diffusing in the cytoplasm. During heat shock, on the other hand, RelA leaves the ribosome for a few minutes and rapidly reassociates upon cellular adaptation (Fig. P1, *Inset*). Our observations suggest that multiple ppGpp synthesis events occur upon RelA dissociation. We refer to this model as "the extended hopping model".

Our experimental approach has several inherent limitations. First, during the course of one experiment we record single molecule trajectories of only one type of molecule at a time (be it ribosomes or RelA). Molecular interactions are then deduced from the resulting change in the diffusion properties. Our approach is made possible by the tremendous size difference between the ribosome and RelA that manifests itself in fundamentally different diffusion characteristics when RelA binds to a ribosome. Simultaneous tracking of both single RelA molecules and single ribosomes is technically feasible, but the enormous cellular ribosomal copy number and the stochastic nature of photoconversion make capturing the interaction of those two fluorescent binding partners during the course of one experiment exceedingly unlikely.

Secondly, our assay is sensitive only to events that change the diffusion properties of our molecule of interest; it does not detect ppGpp synthesis per se. Nor can tracking inactive, ribosome-bound RelA reveal whether the ribosome is idle or actively engaged in translation.

Finally, the fusion of an enzyme with a fluorescent GFP variant may interfere with its functionality. Control experiments prove that C-terminally labeled RelA can functionally replace unmodified RelA in vivo. However, to date there are virtually no alternatives for stoichiometric fluorescent labeling in vivo other than using GFP-fusions. The development of smaller, brighter, more photo-stable in vivo labels will allow single molecule in vivo kinetic studies covering broader time scales.

The current work has several implications. Based on our in vivo approach we propose a modified model for the catalytic mechanism of RelA, an "extended hopping model," which can now be tested by other methods, such as bulk biochemical investigations. Further, this method can now be applied to many other cellular systems that involve regulatory or repair enzymes that bind to other intracellular target sites, such as DNA, RNA, membranes, or protein complexes. Any intracellular reaction that involves target search will necessarily involve changes in the diffusion characteristics of the enzyme of interest and hence can now be studied in living cells. Suitable in vitro systems are often lacking, especially for "hub" proteins that interact with a multitude of binding partners. A direct mechanistic study of the binding kinetics to such hubs is now feasible. Finally, by recording individual diffusion trajectories, similar to our approach for RelA during heat shock, it is possible to follow cellular adaptation responses in real time, which creates a useful platform for mechanistic studies of cellular physiology.

1. Potrykus K, Cashel M (2008) (p)ppGpp: Still magical? *Annu Rev Microbiol* 62:35–51.
2. Elf J, Li GW, Xie XS (2007) Probing transcription factor dynamics at the single-molecule level in a living cell. *Science* 316:1191–1194.
3. Manley S, et al. (2008) High-density mapping of single-molecule trajectories with photoactivated localization microscopy. *Nat Methods* 5:155–157.
4. Wendrich TM, Blaha G, Wilson DN, Marahiel MA, Nierhaus KH (2002) Dissection of the mechanism for the stringent factor RelA. *Mol Cell* 10:779–788.
5. Montero Llopis P, et al. (2010) Spatial organization of the flow of genetic information in bacteria. *Nature* 466:77–81.