Quantifying enzyme activity in living cells

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For over a century, enzymatic activity has been studied in vitro, assuming similar activity in the crowded cellular milieu. Here, we determined in real time the catalytic activity of TEM1β-lactamase inside living cells and compared the values to those obtained in vitro. We found the apparent in vivo catalytic efficiency, $k_{cat}/K_m$, to be lower than in vitro, with significant cell-to-cell variability. Surprisingly, the results show that inside the cell the apparent catalytic efficiency decreases, and $K_m$ increases with increasing enzyme concentration. To rationalize these findings, we measured enzyme and substrate diffusion rates in the cell and found the latter to be slower than expected. Simulations showed that for attenuated diffusion the substrate flux becomes rate-limiting, explaining why reaction rates in vivo can be independent on enzyme concentrations. The octanol/water partition of the substrate is 4.5, which is in the range of Food and Drug Administration–approved drugs. This suggests substrate-limited reaction rates to be common. These findings indicate that in vitro data cannot be simply extrapolated to the crowded in vivo environment.

Enzymatic reaction mechanisms have been studied in the test tube for over 100 years, with most studies performed using purified enzymes provided with excess amounts of substrate and following product formation over time. Combining these experiments with a solid mathematical procedure for their analysis (as introduced by Michaelis and Menten (1) and extended by Briggs and Haldane (2, 3)) has yielded a profound understanding on how enzymes operate under various conditions. However, in vitro measurements of $k_{cat}$ and $K_m$ may be less relevant in the cell because they mostly represent the initial rate of the reaction under substrate saturation and negligible levels of product (4). This underrepresents factors like limiting substrate, crowding, nonspecific binding, cellular activators or inhibitors, and more. To address these complications, measurements of enzymatic activity have been performed in various crowded environments (reviewed in Ref. 5). These experiments showed that in some cases enzymatic activity is affected by crowding, particularly if the crowder induces conformational changes (6, 7) or affects the oligomeric state of the protein (8–11). A drawback of these studies is that bulk experiments in artificial crowders only partially mimic the complex intracellular environment (12, 13). To bridge the gap, new technologies to study protein activity, stability, and folding within the cell have been developed (14–20). Overall, these experiments have supported the notion that qualitatively, activity in the cell mimics that seen in vitro, but with quantitative variations. Although direct studies measuring the enzymatic parameters, $K_m$, $k_{cat}$, and $k_{cat}/K_m$, in the cell have not been performed, they have been estimated by several different methods. One was to use in vivo–like conditions (21, 22), which resulted in some cases in altered $V_{max}$ values. A second method was by extracting nanoliter volumes from the cell, thus measuring enzymatic activity in extract from individual cells. This showed highly heterogeneous activity of α-glucosidase II (23). A third approach was using omics data, inferring in vivo catalytic rates of enzymes from the flux carried by the enzyme and the enzyme copy number. This resulted in an estimate of catalytic activity in the cell being within a range of 5-fold from the in vitro measured values (24).

The estimates described above assume that all the cells in the same culture are equal. However, cell-to-cell variation originating from changes in the chemical identity of the enzyme (e.g. post-translational modifications) and variations in the enzyme concentration, its oligomeric state, cell density, or composition have been reported (25–28). For example, we and others have demonstrated that diffusion within cells, which relates to soft interactions and crowding, varies by up to 2-fold between individual cells (20).

Here, we studied the enzymatic activity of the enzyme TEM1β-lactamase (β-lac)$^2$ in living HeLa cells. β-lac cleaves β-lactams by a multistep mechanism analogous to the mechanism of serine proteases (29–32). This enzyme is naturally limited to prokaryotes; thus any substrate cleavage in HeLa cells is expected to arise from the transfected TEM1 gene. The enzymatic activities of β-lac has been studied in detail, and a fluorogenic substrate exists. Thus, it is a good model systems for measuring enzymatic reaction constants in vivo and comparing them to in vitro measurements. Surprisingly, we found a very large cell-to-cell variability in apparent catalytic efficiencies between cells. Moreover, mean catalytic efficiency differed substantially between in vivo and in vitro measurements. Simulations and diffusion measurements suggest that substrate attenuation by the in vivo milieu is at least partially to blame for the observed differences. These findings suggest that in vitro data cannot be simply extrapolated to the in vivo environment.

1 The abbreviations used are: β-lac, β-lactamase; ROI, region of interest; M–M, Michaelis–Menten; FRAP, fluorescence recovery after photobleaching; FDG, fluorescein di-β-o-galactopyranoside; RDG, resorufin-β-o-galactopyranoside.

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This article contains Supplemental Figs. S1–S4 and Movie S1.

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Results

Measuring enzyme activity in cells

Experimental determination of catalytic constants requires enzyme and substrate/product concentrations to be known. CCF2 is a fluorogenic substrate of β-lac, which emission peak shifts from 520 to 447 nm upon cleavage (supplemental Fig. S1A). A calibration curve relating product fluorescence with its concentration is shown in supplemental Fig. S1B. Calibration was done under the microscope using the same setup as for in-cell measurements but performed in a drop where the substrate concentration is controlled. To measure the β-lac concentration, the enzyme was fused to mCherry without affecting its catalytic efficiency (as determined in vitro using purified proteins). The amount of β-lac was then calculated from the fluorescence intensity of mCherry (excitation, 559 nm; emission, 575–675 nm) according to a calibration curve (supplemental Fig. S1C). The fused protein mCherry-β-lac was uniformly expressed in HeLa cells by transient transfection of the gene (Fig. 1A, mCherry channel). We have previously shown that transiently expressed CFP-β-lac in HeLa cells is fully active, because it binds its inhibitor protein-BLIP to completion (20).

At time 0, CCF2 was microinjected into the cytoplasm and product formation was followed with a confocal microscope simultaneously at the mCherry channel (excitation, 559 nm; emission, 575–675 nm) and the CCF2 product channel (excitation, 405 nm; emission, 425–475 nm; Fig. 1A). In the absence of β-lac, no substrate degradation or product accumulation was observed (Fig. 1B). The high CCF2 intensity seen outside the cell at time 0 is due to constant release of material by the injector to the media and has no bearing toward the CCF2 injected into the cell, because CCF2-FA (Free Acid) is cell-impermeant. ~2 s after injection the substrate was homogenously distributed throughout the cytoplasm, and within 1–3 min it was fully converted to product. In the recorded images a region of interest (ROI; yellow circles in Fig. 1A) within the cytoplasm was selected in the individual cells for analysis (Fig. 1B). Only cells where the enzyme decreased by less than 20% during the time of recording were chosen for analysis (implying that the cell is intact after injection). In the absence of β-lac, no substrate degradation or product accumulation was observed (Fig. 1B).

Determination of catalytic constants

The standard procedure to calculate $k_{\text{cat}}$, $K_m$, and $k_{\text{cat}}/K_m$ values is by measuring initial reaction rates $[V_0]$ (assuming steady state and excess of substrate), plotting them versus initial substrate concentration $[S_0]$ and fitting the values using the Michaelis–Menten (M–M) equation. However, this method assumes homogeneous measurement conditions, which may not be the case for individual cells, and requires a large range of substrate concentrations, which is difficult to obtain inside cells. Alternatively, one can analyze individual progress curves by regression analysis modeling the M–M approximations by simulation (33). In the later, excess of substrate is not assumed, and each cell is individually analyzed. Progress curves for β-lac catalysis in vitro at different substrate (CENTA) concentrations, and their fitted catalytic constants are shown in supplemental Fig. S2A. Supplemental Fig. S2B shows the catalytic constants for the same data plotting $V_0$ versus $[S_0]$ fitted by the M–M equation. The calculated $k_{\text{cat}}$, $K_m$, and $k_{\text{cat}}/K_m$ values obtained from the individual progress curves are within the experimental uncertainty of fitting $V_0$ versus $[S_0]$ using the M–M equation.

Using the methodology described above (and elaborated on in Ref. 33), we determined apparent $k_{\text{cat}}/K_m$ values for CCF2 catalysis from individual cells transfected with TEM1 (Fig. 2A, first column). In comparison, the second and third columns show data obtained from analyzing the enzymatic activity within a drop of eukaryotic cell extract using the same microscope settings as for in-cell measurements (see also supplemental Fig. S2C for raw data). In the second column, the source of
Quantifying enzyme activity in living cells

- **Beta-lactamase was from the cytoplasmic cell extract of transiently transfected HeLa cells (with the concentration determined by mCherry intensity), whereas in the third column purified recombinant beta-lactamase produced in Escherichia coli was added to the drop (at a defined concentration). For the second and third columns, we determined the enzyme reaction constants from single progress curves as well as by fitting [Vmax] versus [S] using the M–M equation (supplemental Fig. S3, A and B). Examples of single progress curves of reactions in a drop under the microscope and within cells are shown in supplemental Fig. S3, C and D, respectively.**

- **Comparing apparent $k_{cat}/K_m$ values obtained from the different experimental setups (Fig. 2A) shows that the spread of apparent catalytic constants between individual live cells is much larger than that observed in vitro. In addition, the mean value obtained from in-cell experiments is different from the mean values determined in cell extract.**

The effects of mutations of beta-lactamase in vitro and in vivo

Two beta-lactamase mutations were evaluated for their catalytic activity. R244Q was reported to strongly reduce its activity, whereas the effect of G238S is minor. In vitro evaluation of R244Q, using CENTA as substrate shows a 500-fold reduction in $k_{cat}/K_m$. For G238S, a 2–3-fold reduction $k_{cat}/K_m$ was measured (Fig. 2B). The corresponding catalytic constants from M–M fitting of $V_{max}$ versus $S$ are shown in the inset of Fig. 2B.

In-cell measurements of these two mutants (Fig. 2C) are in line with the in vitro results, with the catalytic efficiency of the R244Q being strongly reduced, whereas the effect of the G238S mutation was small. Notably, the magnitude of the effect of the R244Q mutation was different in vitro and in vivo, with the mean apparent $k_{cat}/K_m$ value being reduced by only 25-fold in vivo. Possible explanations for this difference in magnitude can be the different substrate (CENTA versus CCF2) used, the very high $K_m$ of this mutant (which >100-fold above the substrate concentrations used in vivo), or the different environment.

In vivo variation is related to enzyme concentration

The results presented above of the difference between apparent enzymatic efficiency in vitro and in vivo are surprising and require explanation and verification. To address whether the cell-to-cell variation in apparent $k_{cat}/K_m$ is due to data quality or variations within the same cell, we separately analyzed three similar ROIs within the cytoplasm of the same cell (Fig. 1A). Then we compared the variance between the three ROIs to the variance between cells (Fig. 3A). The results clearly show that within the same cell apparent $k_{cat}/K_m$ values are constant, whereas they vary greatly between cells. Next, we compared apparent $k_{cat}/K_m$ values for measurements done on the same day on three different ROIs with those done on different days (Fig. 3B). Clearly, the mean and distribution of values for the seemingly same measurements varied between days, whereas the mean was the same between ROIs on the same day. To try to define the source of cell-to-cell variation and the reason for the difference between the in vitro and in vivo apparent catalytic efficiencies, we plotted beta-lactamase concentrations within the individual cells versus the calculated $k_{cat}/K_m$ for the same cell (Fig. 3C).

To our surprise, we got a tight relation ($r = 0.85$) between these two values with a slope of $-1$. This suggests that the apparent catalytic efficiency of beta-lactamase is decreasing with increasing enzyme concentration. Fig. 3C also includes the apparent catalytic efficiency of the two beta-lactamase mutants: G238S and R244Q, as well as beta-lactamase activity measured in the presence of NaN3. The beta-lactam specific activity scales with concentration for these measurements similarly to wild-type beta-lactam in living cells (NaN3 is a cytochrome oxidase inhibitor that will stop all cellular functions without affecting its structure). The R244Q data are shifted relative to the other data because of lower apparent catalytic efficiency of this mutant. In Fig. 3D, we compare apparent $k_{cat}/K_m$ values to $V_{max}/K_m$ values for the same cells. Clearly, the variance between cells is much smaller when the enzyme concentration is not taken into account (in $V_{max}/K_m$).
Moreover, in vivo variance for \( V_{\text{max}}/K_m \) is similar to that obtained for measurements done in cell extract under the microscope.

An anti-correlation between concentration and catalytic efficiency indicates that the reaction is independent on the enzyme concentration. A direct verification that the in vivo reaction rates for \( \beta\)-lac do not scale with \([E]\) was obtained by plotting the experimentally measured progress curves from nine individual cells with \([E]\) ranging from 0.2 to 4.3 \(\mu\text{M}\) and \([S_0]\) ranging from 6 to 35 \(\mu\text{M}\) (Fig. 3E) and normalizing the raw data by \([S_0]\). This resulted in the nine progress curves to perfectly overlay with one another, despite the 21-fold difference in enzyme concentration (Fig. 3F). These results clearly show that CCF2 catalysis in vivo does not scale with \( \beta\)-lac concentration but only with substrate concentration.

This raises the question of whether the catalytic efficiency of \( \beta\)-lac is inherently concentration-independent. To answer this question, we performed stopped-flow in vitro measurements varying \([E]\) between 5 and 80 nm. Fig. 4A shows that \( V_0 \) scales linearly with \([E]\) (as expected) with a slope of 1. Furthermore, \( k_{\text{cat}}/K_m \) values for \( \beta\)-lac at different enzyme concentrations as determined by progression curve analysis are the same (Fig. 4B), contradictory to the in vivo results. Finally, we plotted \( k_{\text{cat}}/K_m \) versus in vivo substrate concentrations (Fig. 4C) and found no relation between the two. This suggests that the catalytic efficiency is not dependent on \([S]\).

\( K_m \) but not \( k_{\text{cat}} \) varies with \([E]\)

For a more complete analysis of the dependence of \( k_{\text{cat}}/K_m \) on \([E]\) as shown in Fig. 3C, we plotted the calculated \( k_{\text{cat}} \) and \( K_m \)
values determined for the same cells versus β-lac concentration (Fig. 5, A and B). The lower quality of the progression curves measured within cells relative to in vitro data results in higher uncertainty in $k_{\text{cat}}$ and $K_m$ values (33). Still, one sees clearly that $k_{\text{cat}}$ values are not dependent on $[E]$, with the average $k_{\text{cat}}$ value in vivo being 35 s$^{-1}$, which is within the error of the in vitro value determined in cell extract (55 ± 20 s$^{-1}$; supplemental Fig. S3B). Conversely, $K_m$ scales with $[E]$ with a slope of −1, and its values are mostly higher than measured in vitro in cell extract (117 ± 60 s$^{-1}$; supplemental Fig. S3B).

**Variation in apparent $k_{\text{cat}}/K_m$ and $K_m$ values can be explained by substrate attenuation**

CCF2 is a relatively hydrophobic molecule. The octanol/water partition (logP) for CCF2 is 4.5. This value is in the range of commonly used small molecule drugs orally administrated (which are in the range of 1–6). For a drug to be orally absorbed, it must be hydrophobic enough to partition into the lipid bilayer but not so hydrophobic that it will stay in the bilayer. Thus, drugs tend to weakly associate with hydrophobic cellular components through soft interactions. Soft as well as hard interactions alter diffusion rates (12). Although the effect of the latter scales with the size of the crowder and the diffusing molecule, the former depends on the chemical nature of the molecules. Therefore, to determine whether the enzymes or substrates in this study are attenuated in vivo, we performed fluorescence recovery after photobleaching (FRAP) measurements and determined the rate of recovery ($t_{1/2}$; Fig. 6A) and the fraction recovery 1.4 s after initiation of FRAP (Fig. 6B). As expected, FRAP of mCherry-β-gal with a molecular mass of 606 kDa was the slowest because of its high molecular mass. It is surprising to see that mCherry has a slower FRAP rate than TEM1-mCherry, despite it having only half of the molecular mass of the enzymes. FRAP of TEM1-mCherry was faster than CCF2, suggesting that CCF2 diffusion is indeed attenuated in the cell. This was further evident by calculating the fraction recovery of CCF2, which was on average only 55% versus 76 and 87% for TEM1-mCherry and mCherry. This indicates that a significant fraction of CCF2 is not freely diffusing in the cell. We further inspected FRAP of two additional substrates, fluorescein di-β-D-galactopyranoside (FDG) and resorufin-β-D-galactopyranoside (RDG) (supplemental Fig. S4). RDG had the lowest fraction recovery, whereas recovery of FDG was somewhat higher. FRAP rates for the three substrates were the same, although slower than measured for TEM1-mCherry. Because we did not observe aggregates of any of the substrates we assume them to be retained by weak, mostly hydrophobic interactions all over the cell (as predicted for CCF2 by logP), whereas β-lac and β-gal seem to freely diffuse.

\[
[S]+[E] \rightarrow ES \rightarrow E[P]
\]

**SCHEME 1**

To simulate the effect of substrate attenuation on catalytic efficiency, we used the ProKII software and introduced an interaction of $[S]$ with $[X]$ to the reaction model (scheme 1), where $[X]$ is a generic representation of the soft interactions of $[S]$, preventing it from forming the $[ES]$ complex (Scheme 1).

Fig. 7A shows the simulated curves using Scheme 1, with $[S_0]$ and $[E]$ values taken from the reactions shown in Fig. 3E. For the simulations $k_1$ and $k_{-1}$ were set to 0.05 s$^{-1}$, $k_2$ was set to 10 s$^{-1}$, $k_{-2}$ was set to 900 s$^{-1}$, and $k_{\text{cat}}$ was set to 55 s$^{-1}$, in line with the values obtained in stopped-flow measurements. Under these conditions, the simulated progress curves for the individual cells closely resemble their experimental counterparts (compare Fig. 3E with Fig. 7A). Normalizing the curves in Fig. 7A by $[S_0]$ (as done in Fig. 3F) results in an overlay of the reaction curves (Fig. 7B), similar to what is observed when normalizing the experimental data by $[S_0]$ (Fig. 3F). This shows that indeed for both the experimental and simulated data, the enzyme concentration is not a factor in determining the progress of the
product formation in the case where \([S]\) is attenuated by \([X]\). This observation can be rationalized by the concentration of the free substrate being lower than the enzyme concentration, making the flux of free substrate (from bound to unbound) the rate-limiting reaction step. To further make this point, we simulated two additional reaction schemes. One where \([E]\) is attenuated by \([X]\) (and thus the amount of free enzyme is much lower; Fig. 7, C and D) and the second being the standard M–M reaction without attenuation of either \([E]\) or \([S]\) (Fig. 7, E and F). In both cases, the simulated curves do not resemble the experimental data.

**Discussion**

In this manuscript we determined the catalytic efficiency in eukaryotic HeLa cells for \(\beta\)-lac. To the best of our knowledge, this is the first time that catalytic efficiency was directly evalu-
Quantifying enzyme activity in living cells

Figure 7. Simulating progress curves of β-lac. A, simulating individual progress curves using Scheme 1 with [S₀] and [E] being the same as the experimental curves in Fig. 3E. For the simulation k₁ and k⁻₁ were 0.05 s⁻¹, k₂ was 10 s⁻¹, k⁻₂ was 600 s⁻¹, and k⁺ was 55 s⁻¹. B, normalizing the curves in A by [S₀] (as done in Fig. 3F) results in overlay of the simulated reaction curves. C, simulating individual progress curves with [E] being attenuated by [X]. D, the data in C were normalized by [S₀] (as done in Fig. 3F). E, simulating individual progress curves without any attenuation. F, the data in E were normalized by [S₀]. The simulations were done using ProKII (Applied Photosystems).

ated within living cells. Because of the stochastic nature of gene transfection and our ability to choose cells with a wide range of protein concentrations, we were able to determine catalytic constants in cells with enzyme concentrations ranging from 0.01 to 10 μM and substrate concentration ranging from 1 to 100 μM. These are common concentrations for enzymes and
substrates in the cell (34). Surprisingly, the in vivo determined $k_{cat}/K_m$ values differed from in vitro values, both in magnitude and in variance between the individually analyzed reactions. Further analysis showed this to be a result of $k_{cat}/K_m$ negatively scaling with [E], with the slope of the log-log plot being $-1$. To verify that these results are not related to an intrinsic problem with the β-lac enzyme, we determined that in vitro $k_{cat}/K_m$ is independent on enzyme concentration (and thus reaction rates scale linearly with [E] (Fig. 4A)).

A first clue to explain these results came from FRAP measurements of the CCF2 substrate, the $t_{1/2}$ of which was slower than that measured for mCherry-β-lac despite the 70-fold lower molecular weight of the CCF2 substrate (molecular mass 856 Da versus 50 kDa for the enzyme; Fig. 6A). The slower $t_{1/2}$ suggests that the substrate is partially attenuated by cellular components and thus is not free to engage the enzyme. This becomes even clearer when looking at the fraction recovery after 1.4 s (Fig. 6B), which was 55% for CCF2 versus ~80% for mCherry and mCherry-β-lac. We further determined the FRAP rates and fraction recovery for two additional fluorogenic substrates (FDG and RDG) and found both to be slower than mCherry-β-lac and with lower fraction recovery (Fig. 6A and B). Because the logP value of CCF2 is 4.5, this result should not be surprising, because the molecule is quite hydrophobic. Although logP of 4.5 is high for standard, water soluble compounds, it is within the normal range of Food and Drug Administration–approved small molecule drugs according to the Lipinski rule of five. To further verify that attenuated CCF2 substrate could explain our experimental results, we simulated product formation using the experimentally determined catalytic constants but adding a term of substrate release to Scheme 1. Indeed, the experimental progression curves were perfectly simulated when the substrate was attenuated by the cell (Fig. 7). Performing the simulations with either the enzyme being attenuated or without attenuation did not result in the experimental reaction curves. To obtain further insight into the mechanism allowing for the reaction rate to become independent on β-lac concentration, we performed a simulation of the conversion of 30 μM [S] using two enzyme concentrations: 0.5 and 5 μM (Fig. 8). As can be seen, the progress curves are the same in both cases. The explanation for this is the 5-fold higher free substrate in the case of 0.5 μM [E], which results in the same concentration of [ES] (and thus [P]) in both cases. This also explains why $k_{cat}$ is independent on [E] ($k_{cat}$ is the same for both cases; Fig. 4), whereas the apparent $K_m$ is increasing with higher [E]. It is not that $K_m$ is different in the cell with different [E], but the amount of free substrate scales with [E], resulting in an apparent dependence of $K_m$ and $k_{cat}/K_m$ on [E].

Following CCF2 catalysis in cell extract (in vitro) resulted in similar catalytic constants as measured in buffer (Fig. 2, A and B). This would suggest that CCF2 is not attenuated by the cell extract, as it is in the cell. This can be explained by the >10-fold dilution of proteins in cell extract compared with in vivo and the lack of membranes and nucleic acids in the cell extract.

Overall, these results suggest that the amount of free substrate is much lower than the total substrate, resulting in the rate of substrate release to its free form being the rate-limiting step in dictating the overall reaction rate. An interesting point to consider is that limiting free substrate results in the amount of enzyme being less important. Thus, the availability of substrate will dictate product flux and not the amount of enzyme. In such case, variation in the amount of enzyme will have no effect on catalysis in the cell, providing a substrate-driven rather than an enzyme-driven control on the rate of product formation. There are also other cases where substrate and not enzyme will be limiting. For example, when the substrate is imported into the cell at a certain rate or when the enzyme is part of a metabolic pathway, the slowest (least abundant) enzyme along the pathway dictates the overall flux.

**Materials and methods**

CCF2-FA (free acid) was purchased from Thermo Fisher Scientific (catalog nos. K1039 and M0257), and CENTA was purchased from Calbiochem (catalog no. 219475). All other reagents were purchased from Merck or Sigma-Aldrich at the highest quality level available.

**Protein engineering and purification**

mCherry-TEM1-β-lac from E. coli was constructed by fusing the N terminus of TEM1 (blaTEM1) (excluding the first 23-amino acid leader sequence) to His$_{6}$-mCherry with a 5–amino acid linker (GGSGS) between them. For protein purification, mCherry-TEM1-β-lac and mCherry-β-gal were cloned into the pET9a vector under the control of T7 promoter. E. coli BL21(DE3) cells were transformed with the plasmids and grown in 2YT medium at 37 °C to an optical density of 0.6. Protein expression was induced by addition of 100 μM isopropyl β-d-thiogalactopyranoside. After induction, the cells were grown at 20 °C for 16 h, centrifuged, lysed by sonication, and centrifuged again. For protein purification, the supernatant was centrifuged again. For protein purification, the supernatant was centrifuged again.
Quantifying enzyme activity in living cells

with 0.5 M imidazole. The collected protein fractions were loaded onto a HiTrap Q HP anion exchange column (GE Healthcare) and eluted with a linear salt gradient from 20 mM to 1 M NaCl. Following the ion exchange, the protein was dialyzed against PBS, and size exclusion chromatography was performed to achieve further purification (Superdex 75 26/60 for TEM1 and Superdex 200 10/300 for β-gal, respectively). The purified protein was analyzed by 12% SDS-PAGE stained with Cooomassie stain (Bio-Rad). Protein concentration was determined by 280- and 587-nm absorption. The protein was then aliquoted, flash-frozen in liquid nitrogen, and stored at −70 °C.

TEM1 mutants

The mutations that confer activity toward cephalosporins were introduced into the wild-type mCherry-TEM1 gene cloned into pmaxGFP vector for transient transfection and into pET9a for in vitro expression. Mutagenesis was performed using restriction free cloning (35).

Cell culture

HeLa cells were grown in 35-mm glass-bottomed dishes (MatTek Corporation) in DMEM (Invitrogen) supplemented with 1× pyruvate, penicillin/streptomycin (BioLabs), and 10% fetal bovine serum (Equitech-BIO, Inc.). The cells were subcultured when ~80% confluence was reached using trypsin-EDTA for cell detachment. One day before transient transfection, 2 × 10⁶ HeLa cells in 2.5 ml of DMEM were pipetted into glass-bottomed dishes and incubated overnight. The cells were cultured in humid atmosphere at 37 °C and 5% CO₂. Transient transfection of mCherry-TEM1 and mCherry alone cloned into pmaxGFP vector (Amaza) under the control of CMV promoter and the mCherry-lacZ construct in the pCH110 plasmid (Pharmacia) under the control of SV-40 promoter was performed with jetPEI (Polyplus transfection) according to the manufacturer’s standard protocol. The cells were imaged 24–30 h after transfection. Prior to microinjection, the medium was aspirated, and the fresh medium was supplemented with 25 mM Hepes, pH 7.4. In some cases HeLa cells were also treated with NaN₃ (0.05% w/v) and incubated for 1 h prior to microinjection.

Microinjection

Microinjections were performed using Eppendorf Femtojet microinjector attached to Eppendorf InjectMan N12 micromanipulator. The sample was injected into cells using glass capillaries from Warner instruments and pulled by vertical puller (Narishige). For every measurement, a single pressure pulse was applied to deliver the sample into the cell. Air was administered at 40–80 hPa for 0.3 s. Before being loaded to the capillary, the injected fluorophores were diluted in PBS to final concentrations of 100 and 200 µM. For injection, single cells where mCherry-β-lac could be detected within an area containing morphologically healthy and well connected cells were selected. Before and after microinjection, cell morphology and the membrane integrity were confirmed by visually inspecting the injected cells.

Confocal microscopy

The cells were imaged with an Olympus FluoView IX81 FV1000 IX81 Spectral/SIM Scanner confocal laser-scanning microscope (Olympus GmbH, Hamburg, Germany), using 60× differential interference contrast oil-immersion objective, N.A. 1.35. For kinetic measurements, the fluorogenic β-lac substrate CCF2 was excited at 405 nm using a diode laser at an output power of 1.5 and 0.5% for low and high substrate concentrations. The mCherry fluorescence was excited at 559 nm using a DPSS laser at 10%. In the intact CCF2 molecule, excitation of the coumarin donor at 405 nm leads to FRET to the fluorescein acceptor and emission of green light (36). The TEM1-catalyzed hydrolysis of the substrate separates the donor and the acceptor (supplemental Fig. S1A). Emission was recorded simultaneously from 425 to 475 nm and from 510 to 548 nm, and mCherry emission was recorded sequentially using the spectral detection system. The FRET channel records acceptor emission (510–548 nm) during donor excitation (405 nm). Three channels were used for detecting CCF2 hydrolysis: an acceptor channel (excitation, 405 nm; emission, 510–548 nm), a donor channel (detecting the product formation; excitation, 405 nm; emission, 425–475 nm), and a third channel to visualize the mCherry fluorophore in the cells (excitation, 559 nm; emission, 575–675 nm). Images were collected throughout the reaction with a scan speed of one frame/0.427 s. After microinjection, CCF2 is trapped inside the cells because of its charges. Substrates were injected to cells void of mCherry-labeled enzymes, serving as a control. All image analyses were performed using FluoView software (Olympus), and data analyses were performed using MatLab software.

FRAP

The photobleaching method (line-FRAP) was carried out on the mCherry and mCherry-β-lac transfected cells or after microinjection of different fluorogenic substrates such as CCF2, FDG, and RDG to nontransfected cells. First, HeLa cells were transiently transfected with pmaxGFP (Amaza) for mCherry and mCherry-β-lac expression or with pCH110 (Pharmacia) for mCherry-β-gal expression. The target cell was imaged with the main scanner at 559-nm excitation using 1% of the maximal intensity during bleaching. We have used a 60× differential interference contrast oil-immersion objective lens during all FRAP experiments. To bleach the desired area, a “tornado” of 4 pixels diameter was used in the simultaneous stimulus scanner. The unidirectional lines were scanned with time intervals of 1.47 ms for 1000 times (equivalent to 1.472 s). The number of scans before, during, and after the photobleaching was kept fixed at 10, 42, and 948, respectively. Photobleaching was achieved with the simultaneous scanner laser at 405-nm excitation for 63 ms, using full intensity. The simultaneous scanner moved at a speed of 100 µs/pixel to perform an efficient photobleaching. We have used two simultaneous lasers during the FRAP experiments: one laser (at 405 nm) for photobleaching and another laser for data acquisition. For mCherry and mCherry-β-lac transfected cells, an excitation of 559 nm was used at intensity 5.0% for scanning the images. For RDG, excitation at 514 nm was used with intensity 12%. For FDG and CCF2, excitation at 440 nm was used with intensity of 3% for the later and varied between 3 and 10% for the former. This setup allows for determination of t₁/₂, the time required for the fluorescence intensity in the bleached...
region to recover to 50% of the plateau fluorescence intensity. Fluorescence recovery plots were fitted to a single exponential growth curve.

In vitro measurements

Michaelis–Menten parameters were determined using a stopped-flow spectrophotometer (Applied PhotoPhysics) at 25 °C (if not otherwise stated) in absorbance mode (β-lac). CENTA (10–3000 μM) was hydrolyzed at 25 °C with mCherry β-lac (0.02–1 μM). The hydrolysis of CENTA was monitored by continuously recording the absorbance variation at 405 nm (Δε = +6,400 M⁻¹ cm⁻¹) for 500 s on the stopped-flow for wild-type TEM1 and for TEM1 G238S mutant. All TEM1 measurements were carried out at pH 7 in a 50 mM sodium phosphate buffer.

Cell extract measurements

Cytoplasmic HeLa cell extracts (2 and 8 mg/ml) were prepared as described (20). To relate mCherry β-lac fluorescence to concentration, up to 25 μM of purified proteins were diluted in cell extract, placed on 35-mm glass-bottomed dishes (MatTek Corporation), and imaged with the confocal microscope. Calibration curve of the hydrolysis product (hydroxycoumarin) was obtained by plotting fluorescence intensity versus substrate concentration (0.5–20 μM diluted in cell extract) after complete conversion by the enzyme (0.1 μM). Fluorescence intensity 2 μM above the surface was plotted against protein concentration. The measurements were performed with different preparations of cell extracts, yielding similar fluorescence intensities per micromolar. For enzyme kinetic measurements, the purified recombinant enzyme diluted in cell extract or the cytoplasmic mCherry-β-lac were placed on a glass-bottomed dish. The interaction was initiated by rapidly pipetting an equal volume of the fluorescent substrate CCF2 in cell extract, respectively. Measurement setup was identical to in-cell measurements. For the preparation of cytoplasmic mCherry-β-lac, the cells were grown on a 14-cm vessel and transiently transfected using jet-PEI transfection reagent.

Progress curve analysis

Single progress curves were analyzed with a Matlab program that simulates M–M-like kinetics and subsequently aligns the simulation with experimental data (33). The simulation is performed time-stepwise with an adaptive algorithm to reach high accuracy at critical regions. For alignment a least-square parameter estimator is utilized with a trust-region-reflective algorithm. An optional diminishing rate or constant is added for exponential or linear leakage respectively. The Matlab tool and a description of how to use it can be found online at the Weizmann Institute website.

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