Fluctuations and Quality of Control in Biological Cells: Zero-Order Ultrasensitivity Reinvestigated

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ABSTRACT Living cells differ from most other chemical systems in that they involve regulation pathways that depend very nonlinearly on chemical species that are present in low copy numbers per cell. This leads to a variety of intracellular kinetic phenomena that elude macroscopic modeling, which implicitly assumes that cells are infinitely large and fluctuations negligible. It is of particular importance to assess how fluctuations affect regulation in cases where precision and reliability are required. Here, taking finite cell size and stochastic aspects into account, we reinvestigate theoretically the mechanism of zero-order ultrasensitivity for covalent modification of target enzymes (Goldbeter and Koshland (1981) *Proc. Natl. Acad. Sci. USA.* 78:6840–6844). Macroscopically, this mechanism can produce a very sharp transition in target concentrations for very small changes in the activity of the converter enzymes. This study shows that the transition is much more gradual in a finite cell or a population of finite cells. It also demonstrates that the switch is exactly analogous to a thermodynamic phase transition and that ultrasensitivity is inevitably coupled to random ultravariation. As a consequence, the average response in a large population of cells will often be much more gradual than predicted from macroscopic descriptions.

INTRODUCTION

It has long been appreciated (Benzer, 1953; Spudich and Koshland, 1976; Berg, 1978; McAdams and Arkin, 1999) that the finite size of biological cells can introduce a large statistical uncertainty to the concentrations of its constituents. All chemical reactions are probabilistic by nature, and the influence of fluctuations depends critically on the design of the kinetic mechanism. Some negative feedback systems can reduce copy number fluctuations indefinitely, albeit at a high energy cost (Paulsson and Ehrenberg, 1998). For mechanisms that depend nonlinearly on fluctuating entities, molecular-level kinetics, rather than its macroscopic counterpart, is essential not only to describe fluctuations, but also to correctly account for population averages (van Kampen, 1992). This so-called mesoscopic kinetics is based on master equations describing the evolution of probability distributions and is the logical foundation for all macroscopic descriptions (van Kampen, 1992).

Intracellular processes must respond to changes in the intra- or extracellular environment. Sometimes a switch-like mechanism is required where a small change in a signal will lead to, for instance, a complete shutdown of a metabolic pathway. One of the principal mechanisms to achieve such control is through covalent modification, e.g. phosphorylation, of key target enzymes by converter enzymes. If both the enzymes for the modification process and those for its reverse, demodification, are working near saturation, these processes will be zero order, i.e., they will occur at rates that do not depend on the concentration of the target enzymes. Thus, zero-order ultrasensitivity (Goldbeter and Koshland, 1981) is based on opposing fluxes of modification and demodification with near zero-order rates k_1 and k_2, respectively. In a macroscopic perspective, where the numbers of all molecules in the system are infinitely large, and assuming full saturation of converter enzymes, there are only two possible stationary states: when $k_1 > k_2$, all targets are modified, and when $k_1 < k_2$, all targets are unmodified. Thus, a minor change in the environment that influences k_1 or k_2 can change the stationary state completely from one extreme to the other so that, in this limit, the extent of modification is ultrasensitive to such changes and can work as a molecular switch (Goldbeter and Koshland, 1981).

Using master equations, we show here that when zero-order ultrasensitivity is implemented in small systems (living cells) it is invariably coupled to large random fluctuations. Such fluctuations tend to make the average response of the mechanism much less sensitive than could be expected from the macroscopic idealizations that until now have been used to characterize it. We point out that the macroscopic zero-order switch is isomorphic with a thermodynamic phase transition with its characteristic macroscopic fluctuations at the transition point (Haken, 1983). Furthermore, converter enzymes can only be saturated when present in lower concentrations than their targets, and low converter copy numbers imply significantly fluctuating rates of modification and demodification. If these fluctuations are slow, they can make the net modification rate move slowly between positive and negative values so that individual cells drift randomly from having mostly modified to mostly unmodified targets. This phenomenon adds to the gradual disposition of time and population averages and further emphasizes that it is essential to take stochastic aspects into account for biologically relevant descriptions of ultrasensitivity.

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The expected behavior of a whole cell population is considered by taking the appropriate population averages of the results for finite cells. By contrast, the macroscopic description corresponds to a situation where the contents of all cells have been poured together into one single container, more reminiscent of an in vitro experiment than the in vivo behavior. Indeed, a population of independent and finite cells can behave very differently from the macroscopic prediction, even if all concentrations and other parameters are the same and even if the number of cells becomes infinitely large.

It should be stressed that the results of this analysis do not discredit zero-order ultrasensitivity as a general principle for obtaining precise and reliable control of intracellular processes. Rather, it is shown that there are conditions where the mechanism can work with high sensitivity and generate low random variation. Our main aims are instead to identify these conditions and to elucidate those underlying statistical principles that determine what cells can and cannot do. The mesoscopic approach taken here can be applied to a multitude of other intracellular processes and will in many cases be the only way to assess the performance of control circuits in single cells and cell populations.

Macroscopic versus mesoscopic description

The traditional macroscopic description of molecular reactions involves the use of concentrations where molecular amounts change continuously and deterministically. There is no allowance for fluctuations or uncertainties. This description usually works well in very large and homogeneous systems, where molecule numbers can be considered nearly infinite and all molecules have the same environment. The reactions inside a single biological cell are very different. First of all, a biological cell is a finite system where some key components can be present in small copy numbers, e.g., 0–1000 copies for many control proteins and other enzymes. Because all chemical reactions are probabilistic in nature, the consequence of the finite numbers is that descriptions must be at the level of probabilities that a cell contains a certain number of the molecules in question. This is the mesoscopic description where the time evolution of the probabilities is determined from a set of master equations (van Kampen, 1992). Such descriptions can become very intricate when many different kinds of molecules are involved. In this communication we consider mostly the simplest case with a single dimension where only the number of one molecular species changes probabilistically. In this case, it is very simple to calculate the stationary probability distribution P_n that a cell contains n modified enzymes. This stationary distribution could be interpreted as the probability for the momentary number in a single cell. Thus if we were to measure the number at different and well separated time points, with probability P_n we would find n molecules in the cell. Alternatively, P_n could be considered as the probability that a certain cell in a large population of independent cells contains n molecules. Thus the probability distribution can be used also to describe the properties of a population of cells. The averages over the distribution that we calculate below can therefore either be considered as time averages in a single cell, or as the averages over all cells in a population. Similarly, the variance in the number n is a measure of the fluctuations over time in a single cell, or a measure of the differences between cells in a population.

Often one expects that the mesoscopic description will give the same average behavior as the macroscopic one. However, this is not true for processes that are governed by nonlinear terms, and, as we shall see below, zero-order ultrasensitivity is a highly nonlinear process so that the macroscopic description is not even correct for the average behavior. Only in the limit where each cell can be considered infinitely large with negligible intracellular fluctuations will the macroscopic description give the correct average behavior.

In this communication we describe how fluctuations inevitably will blur a response that, in a macroscopic picture, is expected to be sharp and switch-like. We have also recently described in a mesoscopic analysis the effects of fluctuations on a response that is expected to be gradual, e.g., hyperbolic. Surprisingly, in this case fluctuations can make the response much sharper, a phenomenon that we have termed stochastic focusing (Paulsson et al., 2000; Paulsson and Ehrenberg, 2000a).

Macroscopic system

To set the stage, we briefly consider the macroscopic model by Goldbeter and Koshland (1981, 1982). Assume that the total concentration of target proteins that can be modified is C_0, the fraction that is modified is f, the rate of modification is $k_1C_0(1 - f)/(K_M + C_0(1 - f))$, and the rate of demodification is $k_2C_0f/(K_M + C_0f)$. Although the modification and demodification reactions are in general carried out by different enzymes, for simplicity, they have been assumed to have the same Michaelis constant K_M. A change in the fraction f is the response parameter of the mechanism. At the stationary state the flows of modification and demodification are equal:

$$\frac{\alpha(1-f)}{K_M^0 + 1 - f} = \frac{f}{K_M^0 + f} \quad (1)$$

Here $K_M^0 = K_M/C_0$ is a dimensionless Michaelis constant and $\alpha = k_1/k_2$ is the ratio of the maximum rates of the converter enzymes. α depends directly on the concentrations of active converter enzymes, and a change in α serves as the signal in the mechanism that leads to a response in f. When $K_M^0 \ll 1$ so that the enzymatic reactions are saturated, the response will be an abrupt switch from $f \approx 0$ to $f \approx 1$ when the signal is a change from $\alpha < 1$ to $\alpha > 1$ (see

Fig. 1). Such a change in α can be achieved through a small change in activity or concentration of either or both converter enzymes.

The most commonly used discrete sensitivity amplification factor, $A_{f,\alpha}$, is defined as the relative change in f divided by the relative change in α, $A_{f,\alpha} = \Delta f/\Delta\alpha \cdot (\alpha_1/f_1)$ where $\Delta f = f_2 - f_1$ and $\Delta\alpha = \alpha_2 - \alpha_1$ and the subscripts 1 and 2 refer to the before and after values, respectively (Savageau, 1971; Goldbeter and Koshland, 1982). This measure is asymmetric in that a change from α_1 to α_2 gives a different $A_{f,\alpha}$ value than the change back from α_2 to α_1. The kinetic mechanism described here is intrinsically symmetric, which makes the threshold where $k_1 = k_2$ a suitable reference point. We therefore consider only cases where α changes from $\alpha = 1/(1 + \delta)$ to $\alpha = 1 + \delta$, where $0 < \delta < \infty$, and instead use a symmetric sensitivity measure normalized by the reference point $f = 0.5$ and $\alpha = 1$:

$$S_{f,\alpha} = 2\,\frac{\Delta f}{\Delta\alpha} \tag{2}$$

Calculating the sensitivity $S^{80}_{f,\alpha}$ for a response of 80%, $\Delta f = 0.8$, gives

$$S^{80}_{f,\alpha} \approx \begin{cases} 0.09/K^0_M & \text{for } K^0_M \ll 1 \\ 0.18 & \text{for } K^0_M \gg 1 \end{cases} \tag{3}$$

When the converter enzymes are far from saturation, the sensitivity is the same as for a simple Michaelis-Menten mechanism for which one also finds $S^{80}_{f,\alpha} = 0.18$. Macroscopically the sensitivity increases indefinitely with the degree of saturation of the converter enzymes when the reactions become zero-order. Goldbeter and Koshland (1981, 1982) define ultrasensitivity as a response that is much more sensitive to a change in stimulus than a Michaelis-Menten relation, hence the term zero-order ultrasensitivity.

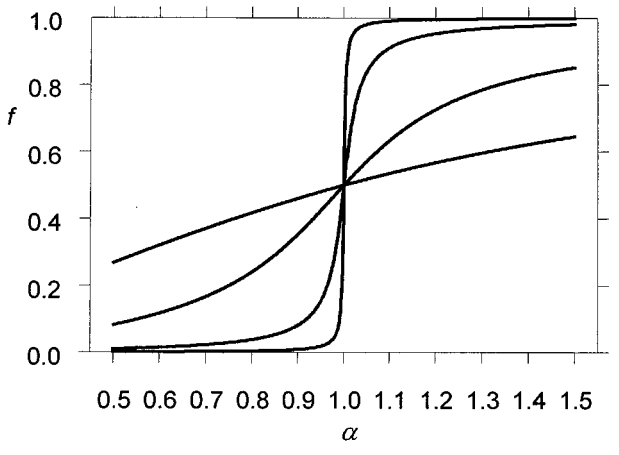

FIGURE 1 The response function, f, of the macroscopic switch with different Michaelis constants, K^0_M. With decreasing sharpness of the curves, $K^0_M = 0.001, 0.01, 0.1, 1$.

Fluctuations in single cells and population averages

The macroscopic analysis above assumes that concentrations change continuously and deterministically without fluctuations and uncertainties. With the more realistic assumption that each cell is finite with N target enzymes out of which n are modified, changes in n are described by the mesoscopic reaction scheme

$$0 \underset{k^d_1}{\overset{k^m_0}{\rightleftharpoons}} \cdots n \underset{k^d_{n+1}}{\overset{k^m_n}{\rightleftharpoons}} \cdots N-1 \underset{k^d_N}{\overset{k^m_{N-1}}{\rightleftharpoons}} N \tag{4}$$

The reaction transition probabilities per time unit for modification and demodification in this birth and death process (van Kampen, 1992) are assumed to be given by the same Michaelis-Menten rates as used in the macroscopic case above:

$$k^m_n = \frac{k'_1(1-f)}{K^0_M + 1 - f}, \quad k^d_n = \frac{k'_2 f}{K^0_M + f} \tag{5}$$

Here $f = n/N$ and k'_1, k'_2 are the maximum rates of the converter enzymes k_1, k_2 (modification and demodification, respectively) multiplied by the cell volume. The scheme (Eq. 4) can be used as the basis for the master equations that describe the time evolution of the probabilities that an individual cell contains n (= 0, 1, . . . N) modified molecules. If the system is a collection of independent cells, the same probabilities define the distribution of molecule numbers across the cells in the population. We first consider the zero-order limit $K^0_M \to 0$, and return to non-zero Michaelis constants further below.

Fluctuations in single cells drastically change population averages

In the limit when $K^0_M \to 0$, the stationary probability distribution (see Appendix) of n modified target proteins is a truncated geometrical, $P_n \propto \alpha^n$ for $0 \le n \le N$. When in addition $\alpha = 1$, so that the scheme (Eq. 4) is an unbiased random walk, the distribution is uniform, $P_n = 1/(N + 1)$ and the uncertainty in the number n is maximal. From these distributions we calculate the average number of modified targets, $\langle n(\alpha)\rangle$, as a function of α. From a macroscopic viewpoint, one expects unlimited sensitivity so that a minimal shift in signal from $\alpha < 1$ to $\alpha > 1$ would result in a shift from $\langle n(\alpha)\rangle \approx 0$ to $\langle n(\alpha)\rangle \approx N$. However, as seen in Fig. 2, for a collection of identical finite systems (cells) the average response function $\langle f(\alpha)\rangle = \langle n(\alpha)\rangle/N$ becomes increasingly less steep as the size N of the individual systems decreases. Indeed, the fact that cells have finite size makes the mechanism highly nonlinear at the boundaries ($n = 0$ or $n = N$), so that the fluctuations in n in single cells can dramatically affect the average behavior of a whole cell population, as discussed further below.

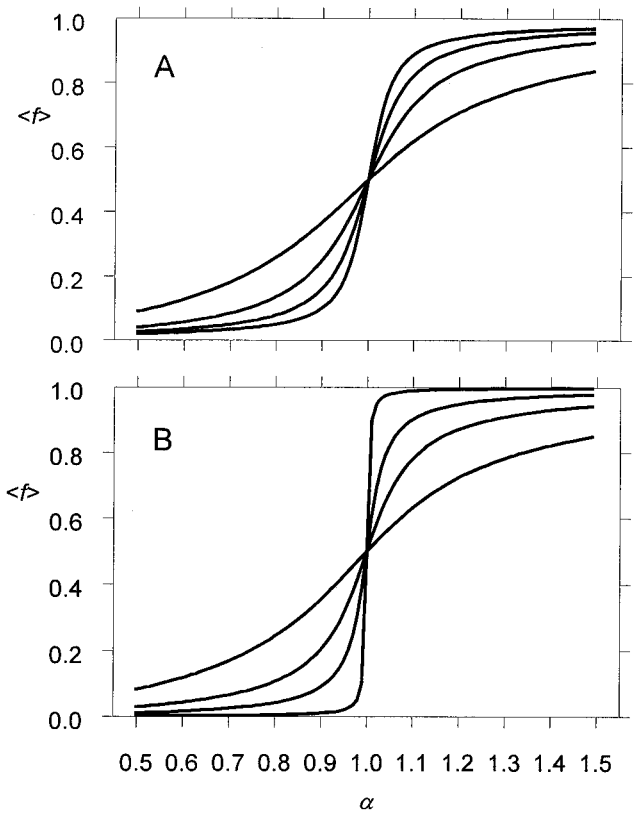

FIGURE 2 The response function, $\langle f \rangle$, of the finite switch. With decreasing sharpness of the curves, $K_M^0 = 0, 0.01, 0.03, 0.1$. (*A*) is for $N = 100$ and (*B*) is for $N = 1000$.

The population-averaged response, $\Delta\langle f \rangle$, can be calculated as outlined in the Appendix (Eqs. A3 and A5). The result for a signal as the symmetric change from $\alpha = 1/(1 + \delta)$ to $\alpha = 1 + \delta$ is shown in Fig. 3. $\Delta\langle f \rangle$ approaches 1 only for sufficiently large signals, where $\delta \gg 1/N$, in contrast to the macroscopic picture with infinitely large cells where $\Delta\langle f \rangle = 1$ for all finite signals δ. At an intermediate point,

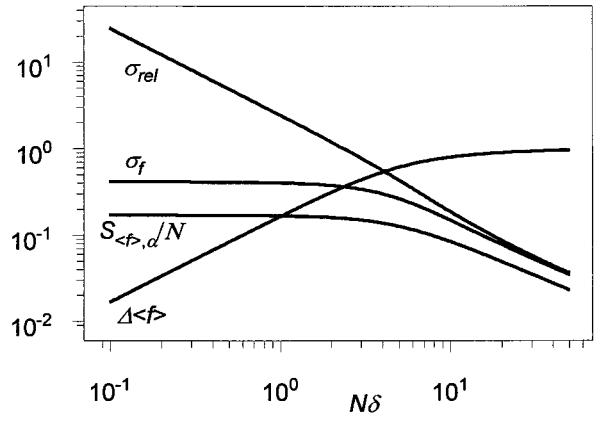

FIGURE 3 The relative response relations, $\Delta\langle f \rangle$, $S_{\langle f \rangle, \alpha}/N$, σ_f, and σ_{rel} for the finite switch with $K_M^0 = 0$, as explained in the text.

one finds that an 80% response, i.e., $\Delta\langle f \rangle = 0.8$, requires the signal (see Fig. 3):

$$\delta^{80} \approx 10/N \tag{6}$$

Similarly, the variance, σ_f^2, between cells in the response Δf can be calculated from the stationary probability distribution (Eqs. A4 and A6). As shown in Fig. 3, σ_f displays an opposite behavior from $\Delta\langle f \rangle$ and reaches a maximum $= 1/\sqrt{6}$ for small signals, $\delta \ll 1/N$. This is the random-walk limit where the number, n, of modified molecules is indeterminate. For large values of δ, the distributions before and after the switch are pushed towards the boundaries and the average shifts from 0 to N. The same force that pushes the distributions to the boundaries also squeezes their variance. Since the distribution cannot move across the boundaries, changes in the variance will by necessity also influence the average. Thus, it is the strong nonlinearity in the single cell system due to the drop to zero in k_n^d and k_n^m at the boundaries $n = 0$ and $n = N$, respectively, that introduces the dependence of the population average on the single cell fluctuations.

The relative uncertainty in the single-cell response, $\sigma_{rel} = \sigma_f/\Delta\langle f \rangle \approx 2/(N\delta)$ (Eq. A7), is also plotted in Figure 3. Thus, $\delta \gg 1/N$ is required for a large response with a small uncertainty. In the macroscopic limit where $N \to \infty$, the relation $\sigma_{rel} = 0$ holds for all finite signals δ. Considered as functions of $N\delta$, the response and its associated uncertainty plotted in Fig. 3 are virtually independent of system size, N. As a consequence, the response and variance considered as functions of the signal δ become extremely sensitive to the system size.

Sensitivity is directly proportional to the single-cell uncertainty

The population-averaged sensitivity can be calculated from the average response using Eq. 2. The relative sensitivity, $S_{\langle f \rangle, \alpha}/N$, is plotted in Fig. 3. Maximum sensitivity occurs for $\delta \ll 1/N$ where it is directly proportional to the size N of the single cell system. Fig. 4 shows the sensitivity as a function of the signal δ for various values of N. To get an 80% response, $\Delta\langle f \rangle = 0.8$, requires $\delta^{80} \approx 10/N$ (Eq. 6) and the corresponding sensitivity is found to be

$$S_{\langle f \rangle, \alpha}^{80} \approx 0.08\, N \text{ if } N \gg 10 \tag{7}$$

Consequently, the macroscopic prediction of unlimited sensitivity when $K_M^0 \to 0$ (Eq. 3) is valid only for infinitely large cells where $N \to \infty$. The response function $\langle f \rangle$ for $K_M^0 \to 0$ for a collection of finite cells (Fig. 2) looks the same as that of the macroscopic system (Fig. 1) with nonzero K_M^0. In fact, introducing a finite size N has a similar effect on the zero-order switch as increasing K_M^0 in an infinite system to a value close to $1/N$; cf. the curves for $K_M^0 = 10^{-2}$ and 10^{-3} in Fig. 1 with those for $K_M^0 = 0$ in Figs. 2 *A* ($N = 100$) and

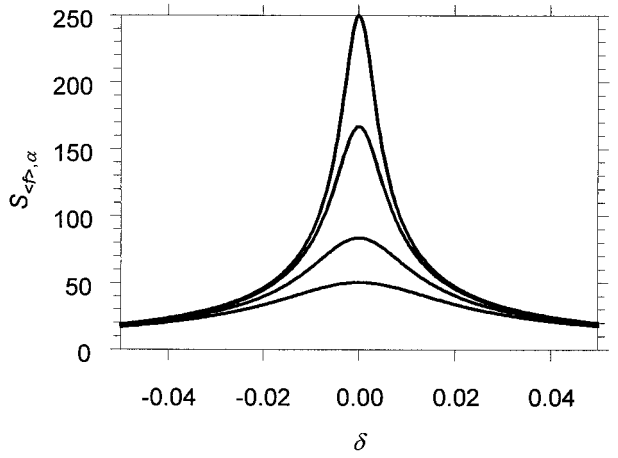

FIGURE 4 The sensitivity, $S_{\langle f \rangle,\alpha}$, of the finite switch with $K_M^0 = 0$ for different values of N. From the lowest to the highest, $N = 300$, $N = 500$, $N = 1000$, and $N = 1500$.

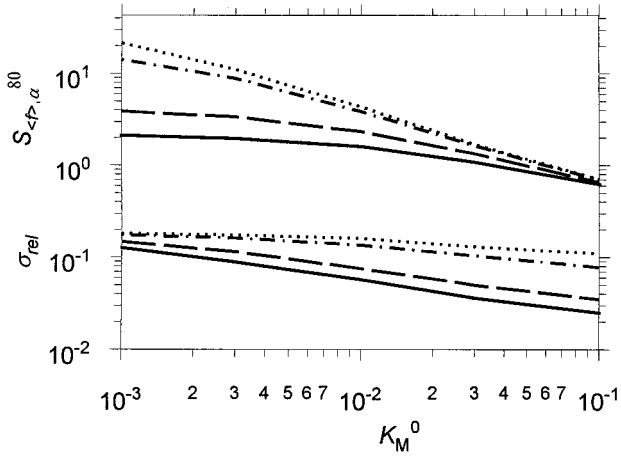

FIGURE 5 The sensitivity, $S^{80}_{\langle f \rangle,\alpha}$ (*upper set of curves*), and the relative fluctuations, σ_{rel} (*lower set of curves*), for the finite switch as a function of K_M^0 for various values of N: $N = 1000$ (——), $N = 500$ (– – –), $N = 100$ (– · – · –), and $N = 50$ (· · ·).

$2B$ ($N = 1000$), respectively. In terms of uncertainty, however, there is no correspondence, since in the macroscopic system the fluctuations in the response Δf are vanishingly small except exactly at the transition point ($\alpha = 1$), where they become macroscopic.

There is a direct proportionality between the average sensitivity $S_{\langle f \rangle,\alpha}$ and the uncertainty, $N\sigma_f$, in the response Δn per cell, which can be expressed as (from Eqs. A6 and A8):

$$\frac{N\sigma_f}{S_{\langle f \rangle,\alpha}} \approx \begin{cases} \sqrt{6} & \text{for } \delta < 1/N \\ \sqrt{2} & \text{for } \delta > 1/N \end{cases} \tag{8}$$

This relationship is nearly invariant to changes both in the size of the cells and in the signal.

Finite K_M^0 further reduces sensitivity

We also calculated the average sensitivity and response for the finite system with non-zero Michaelis constant (Eq. A1). The relationships become more complicated, but the essential consequence of introducing a non-zero K_M^0 is to further decrease both the average sensitivity and the relative single cell uncertainty, as seen in Fig. 5. The numerical results show that the average sensitivity can be approximated roughly as an inverse linear interpolation between Eqs. 3 and 7:

$$S^{80}_{\langle f \rangle,\alpha} \approx \frac{0.08N}{1 + 0.9NK_M^0} \tag{9}$$

which holds for all values of $N \gg 1$ and $K_M^0 \ll 1$. This defines the region of values for N and K_M^0 where ultrasensitivity can be achieved. It should be noted that the term NK_M^0 in the denominator of Eq. 9 is actually independent of N as $NK_M^0 = NK_M/C_0 = K_MV$, where V is the cell volume. Little is gained in average sensitivity by pushing K_MV below 0.5, where $S^{80}_{\langle f \rangle,\alpha}$ is already approximately 70% of its maximum. This suggests $K_MV < 1$ when high sensitivity is selected for. However, K_MV should not be expected to be much smaller than 1, since strong binding is unlikely to evolve without significant functional gain. An *Escherichia coli* bacterial cell has a volume corresponding roughly to 10^9 liters per mole (i.e., 1 molecule per cell corresponds to a concentration of 10^{-9} M). High sensitivity then requires that K_M is of the order 10^{-9} M (denominator in Eq. 9) and that $N > 50$, i.e., $C_0 > 5\ 10^{-8}$ M (numerator in Eq. 9). By contrast, for infinitely large cells, as assumed in macroscopic descriptions, the only requirement to obtain a very sensitive mechanism is $K_M/C_0 \ll 1$ (cf. Eq. 3).

Zero-order ultrasensitivity is equivalent to a phase transition

The properties of zero-order ultrasensitivity bear all the hallmarks of a thermodynamic phase transition (Haken, 1983). In the macroscopic zero-order limit ($N \to \infty$ and $K_M^0 \to 0$), fluctuations (Eq. A6), sensitivity (Eq. A8), and switching time (Eq. A10) are all infinite at the transition point $\alpha = 1$. In finite systems these quantities are all finite, but diverge with increasing system size (cf. Fig. 4).

Although the system is not at thermodynamic equilibrium, the stationary state of the scheme (Eq. 4) is formally identical to an equilibrium state as determined by the forward and reverse rates. If each molecule were modified or demodified independently of the others, the rates would be given by the mass action relations $k_n^m = k_1(N - n)$ and $k_n^d = k_2 n$, and the stationary distribution would be binomial. In contrast, the Michaelis-Menten scheme introduces a strong cooperativity due to competition for converter enzymes. As a consequence, the reaction probabilities per molecule in-

crease the more molecules that have already reacted, pushing the system towards the extreme ends. This apparent cooperativity effect becomes stronger the smaller K_M^0 is, i.e., the closer to zero-order the reaction is.

That the sensitivity to change is proportional to the size of the fluctuations is a general result from statistical thermodynamics. For instance, the sensitivity in heat uptake to a change in temperature is given by the heat capacity, which is directly proportional to the enthalpy fluctuations, $C_p = \partial H/\partial T = kT^2\sigma_H^2$ (e.g., Kittel, 1958). Because large fluctuations arise when there is little resistance to change, it is quite natural that there should be a proportionality between sensitivity and fluctuations. In the system described above, the average sensitivity $S_{\langle f\rangle,\alpha}$ is proportional to the square root of the single cell variance rather than directly to the variance (Eq. 8). This difference appears because, for practical purposes, we are considering the response $\Delta\langle f\rangle$ for a symmetric change of α across the transition point. Defining instead a local sensitivity as $s_\alpha = \partial\langle n\rangle/\partial \ln \alpha$, one finds $s_\alpha = \sigma_n^2$ for all values K_M^0 in full analogy with thermodynamic phase transitions. In the macroscopic limit with $K_M^0 \to 0$, this local sensitivity tends to infinity at the transition point. The analogy with a phase transition is more than superficial. A solid-liquid transition can also be described in a very simple model as a series of zero-order processes, exactly like Scheme 4 with $K_M^0 = 0$. The rates at which liquid molecules join or leave the solid phase depend primarily on the size of the interface between the two phases and not on the number of molecules present in each phase.

The strict mathematical divergence for $\alpha = 1$ in the limit $N \to \infty$ and $K_M^0 \to 0$ is of little consequence for the functioning of the switch. The main point is that the mechanisms behind the mathematical divergences are at play and determine the properties of the switch also when $N < \infty$ or $K_M^0 > 0$. This can be seen in the relationships between sensitivity, fluctuations, and time scale, Eqs. 8 and A11. It can also be noted that the average response function in an infinite population of finite cells is well defined at $\langle f\rangle = 1/2$ for $\alpha = 1$, although the state of each cell is indeterminate. In the macroscopic picture (infinite cells), however, the expected f is totally indeterminate with $0 < f < 1$ at the transition point $\alpha = 1$.

In principle, zero-order ultrasensitivity can appear in systems at equilibrium, as exemplified by the solid-liquid transition. The main determinant is the apparent cooperativity of the zero-order reactions. However, for the covalent modification mechanism considered above, it is only when the converter enzymes are irreversible and energy-driven that this competition can come to bear. In fact, the Michaelis-Menten equations used, Eqs. 1 and 5, are valid only for irreversible enzymes. A true equilibrium would require that the fluxes across each enzyme are reversible and balanced. In this case, the distribution between modified and unmodified targets will be determined simply by an equilibrium constant, K_{eq}, such that $f = n/N = K_{eq}/(1 + K_{eq})$. Thus zero-order ultrasensitivity with covalent modification is possible only with energy-driven opposing fluxes and would disappear at equilibrium (LaPorte and Koshland, 1983).

The breakdown of the law of large numbers and the inconsistency of macroscopic kinetic descriptions near a phase transition have been studied for decades (Matheson et al., 1975; Nicolis and Turner, 1977). The present work shows that it may be of great importance also in central intracellular regulatory processes and that it appears in a very simple and celebrated reaction scheme that (deceptively) appears to be without nonlinear reaction rates.

Limited numbers of converter enzymes and fluctuating rates

First it can be noted that the numbers, N_E, of converter enzymes in single cells (for simplicity N_E is assumed to be the same for modification and demodification enzymes) must be much smaller than the number of targets, n or N-n, respectively, to obtain saturated reactions. When the number of targets falls below N_E, the reactions are no longer zero-order. Fig. 6 *A* shows the behavior of the zero-order switch for $N = 1000$ and different values of N_E. This suggests that $N_E/N < 0.1$ is required for ultra-sensitivity, just as for the macroscopic switch (1). However, N_E cannot be too small without slowing down the switching time, Eq. A11.

Up to now we have dealt with the internal noise in target numbers that arises due to the random walk character of the kinetic mechanism. However, the unavoidable noise in the signal itself must also be accounted for. Because the zero-order rates, k_1 and k_2, are proportional to the numbers, N_E, of converter enzymes, the signal, $\alpha = k_1/k_2$, will fluctuate if these numbers fluctuate. When these fluctuations are slower than the response time of the switch, the switch will fluctuate along with N_E. Consequently, the magnitude of a signal must be larger than the natural fluctuations in N_E, or the entire switch will fluctuate randomly. Thus if these fluctuations are assumed to be Poissonian, the signal must be $\delta > 2/\sqrt{N_E}$. Fig. 6 *B* shows the average response function of the switch assuming independent Poissonian fluctuations in both classes of converter enzymes. In the extreme of very slow enzyme fluctuations, cells can behave very differently, some with the switch on and others off, contributing to the individuality of single cells (Spudich and Koshland, 1976) in a population. In fact, the behavior would then be very close to the bi-stable stochastic switch suggested for bacteriophage lambda (Arkin et al., 1998).

Thus, even if the switch is expected to behave macroscopically in terms of target enzymes, this behavior can be severely corrupted by fluctuations in the number(s) of converter enzymes. With $N_E < N/10$ from Fig. 6 *A* and $\delta > 2/\sqrt{N_E}$ from Fig. 6 *B*, this introduces another limit on the

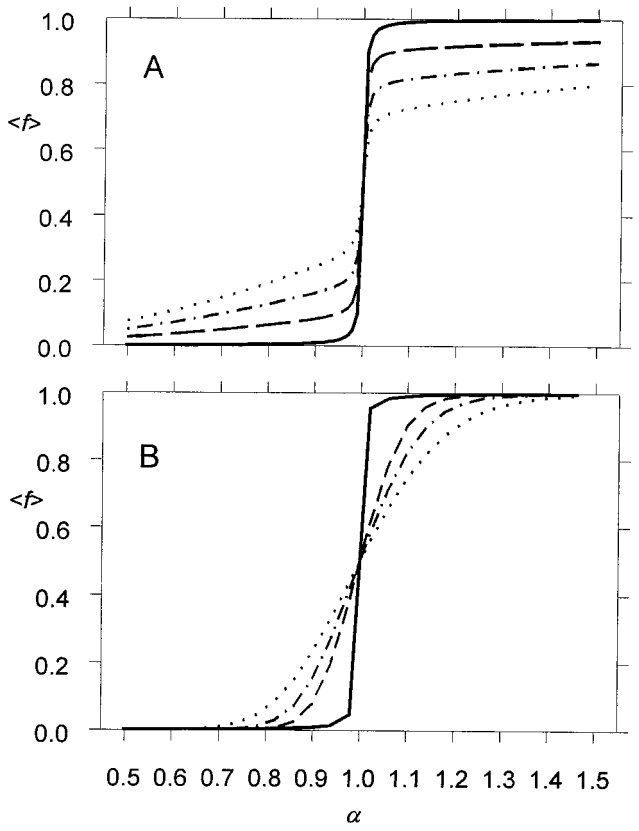

FIGURE 6 Influence from converter enzyme numbers. (*A*) shows the response function when influenced by the first-order regions where the number of substrates is lower than the number of converter enzymes for $N = 1000$ and $K_M^0 = 0$. For simplicity, modification and demodification enzymes are both assumed present at the same number N_E. $N_E \ll N$ (——), $N_E = 100$ (– – –), $N_E = 200$ (– · – · –), $N_E = 300$ (· · ·). (*B*) shows the response function when influenced by fluctuations in the number of converter enzymes. The basic switch is with $K_M^0 = 10^{-3}$ in the macroscopic case, or $K_M^0 = 0$ and $N = 1000$ in the finite case (cf. Figs. 1 and 2 *B*). The *solid line* is the result without converter fluctuations. With decreasing sharpness of the curves, the Poissonian fluctuations in enzyme numbers used in the other curves are 5% (– – –), 7% (– · – · –), and 10% (· · ·), which in the finite case correspond to $N_E = 400$, 200, and 100. The first-order effects shown in (*A*) are not accounted for in this graph.

average sensitivity:

$$S^{80}_{\langle f\rangle,\alpha} \approx 0.8/\delta < 0.4\sqrt{N/10} \approx 0.12\sqrt{N} \qquad (10)$$

This is a more severe restriction than that of Eq. 7. Unless the natural fluctuations in enzyme numbers can be suppressed, it suggests that $N > 1000$ is required for a secure operation of the ultrasensitive switch. It should also be stressed that this conclusion is based on the very conservative Poissonian estimate of copy number fluctuations. Proteins can generally be expected to display far more significant fluctuations (Berg, 1978; McAdams and Arkin, 1997) and the restriction of Eq. 10 can then be far more severe.

An alternative way to avoid these restrictions is to make the fluctuations in enzyme numbers much faster than the response in target numbers. In that case, the switch would sense only the average number of converter enzymes and the fluctuations would not matter. This could be achieved either by slowing down the switch, e.g., with low k_{cat}, or by speeding up converter fluctuations, e.g., with a high turnover. Possibly, the enzyme fluctuations could also be speeded up by retaining a buffer of inactive enzymes in a dynamic equilibrium with active ones. In any case, avoiding the randomizing impact of natural fluctuations in the converter enzyme numbers may incur a high metabolic cost.

CONCLUSIONS

Zero-order ultrasensitivity is a celebrated phenomenon suggested for target modifications carried out by saturated enzymes. The sensitivity arises because the resistance to change is negligible when both formation and elimination follow approximately zero-order kinetics. However, low resistance to change is also generally coupled to large random fluctuations, calling for a molecular-level kinetic analysis. Here we formulated a simple model of a zero-order modification scheme in terms of chemical master equations. The analysis shows how ultrasensitivity is related to random ultravariation and that zero-order ultrasensitivity is isomorphic, with a phase transition where both sensitivity and fluctuations are infinite at the transition point (Haken, 1983). It also shows that macroscopic theory can be far off the mark in its estimate of the average sensitivity due to the hidden nonlinear character of this mechanism and that a high average sensitivity requires a large number of target molecules per cell.

Zero-order ultrasensitivity requires saturated modification reactions, i.e., that the converter enzymes are present in much lower numbers than their substrates. A limited number of converters inevitably results in significant random fluctuations. When these stimulus fluctuations are slow compared to the response, individual cells will randomly jump between the two extremes. Seen as a time or population average, the switch can then again be significantly more gradual than expected from the macroscopic viewpoint.

These effects are of direct relevance to intracellular processes since individual cells often contain enzymes present in low copy numbers. Accordingly, a system that displays strong zero-order ultrasensitivity in vitro is not necessarily a candidate for strong ultrasensitivity under the same conditions in vivo. Notably, for the in vitro situation a high sensitivity requires only that $K_M/C_0 \ll 1$, whereas in vivo there are separate requirements for the concentration, C_0 (or the number N), and the Michaelis constant, K_M. We found that the sensitivity in the mesoscopic picture, Eq. 9, approaches the macroscopic description, Eq. 3, if $K_M V \gg 1$ and $N \gg 1$. On the other hand, maximal sensitivity in the mesoscopic picture is reached when $K_M V < 1$, and then ultrasensitivity is possible only if the number of target enzymes, N, is greater than 100 or so. Clearly, if the volume

V of individual cells (or reaction compartments) is large, e.g., like that of eukaryotic cells, the macroscopic limit will be reached more readily. If the numbers of converter enzymes are fluctuating slowly, there are more severe restrictions on the size of the system; in the case of Poissonian number fluctuations we found that ultrasensitivity would be possible only if the number of target enzymes exceeds 1000 or so. In some of the systems where ultrasensitivity has been studied experimentally (LaPorte and Koshland, 1983; Ferell and Machleder, 1998), the number of enzyme molecules involved is much larger than this.

Zero-order ultrasensitivity is of importance also beyond its suggested role as a control mechanism. Similar dynamics can be expected also in many other biosynthetic reactions with opposing fluxes of synthesis and utilization, and zero-order effects may in fact be hard to avoid in many cases, as has been demonstrated in the case of plasmid copy number control (Paulsson and Ehrenberg, 1998, 2000b). Thus large fluctuations in pool sizes can always be expected for molecules that are synthesized and utilized by near-saturated enzymes. Such systems will also display phase-transition-like behavior.

APPENDIX

At the stationary state there is no net flow across any of the steps in the scheme (Eq. 4). Thus $k^{\mathrm{m}}_{\mathrm{n}}P_{\mathrm{n}} - k^{\mathrm{d}}_{\mathrm{n+1}}P_{\mathrm{n+1}} = 0$, and the probabilities P_{n} that there are n modified proteins in the system can be calculated recursively from $n = 0$.

$$P_{\mathrm{n}} = P_0 \prod_{\mathrm{n}=1}^{\mathrm{N}} \frac{k^{\mathrm{m}}_{\mathrm{n-1}}}{k^{\mathrm{d}}_{\mathrm{n}}} \quad \text{for } n = 1, 2, \ldots N \tag{A1}$$

P_0 can be determined from the normalization condition that the total probability mass sums to one. In the limit where the Michaelis constants are very small so that the enzymes are saturated, Eq. A1 simplifies to a truncated geometric distribution:

$$P_{\mathrm{n}} = \frac{1-\alpha}{1-\alpha^{\mathrm{N+1}}}\,\alpha^{\mathrm{n}} \quad \text{for } n = 0, 1, \ldots N \tag{A2}$$

where $\alpha = k_1/k_2$. The average and the variance of this distribution are functions of α

$$\langle n(\alpha)\rangle = \frac{\alpha}{1-\alpha} - \frac{(N+1)\alpha^{\mathrm{N+1}}}{1-\alpha^{\mathrm{N+1}}} \tag{A3}$$

$$\sigma^2_{\mathrm{n}}(\alpha) = \frac{\alpha}{(1-\alpha)^2} - \frac{(N+1)^2\alpha^{\mathrm{N+1}}}{(1-\alpha^{\mathrm{N+1}})^2} \tag{A4}$$

The response, i.e., the change $\Delta\langle n\rangle$ in $\langle n\rangle$ when α changes from $\alpha = 1/(1+\delta)$ to $\alpha = 1+\delta$, can be calculated from Eq. A3 as $\Delta\langle n\rangle = \langle n(1+\delta)\rangle - \langle n(1/(1+\delta))\rangle$. The variance in the response will be $\sigma^2 = \sigma^2_{\mathrm{n}}(1+\delta) + \sigma^2_{\mathrm{n}}(1/(1+\delta))$ from Eq. A4. These results can be calculated generally, and in the limits of small and large signals one finds

$$\Delta\langle f\rangle = \frac{\Delta\langle n\rangle}{N} \approx \begin{cases} N\delta/6 & \text{for } \delta < 1/N \\ 1 & \text{for } \delta > 1/N \end{cases} \tag{A5}$$

$$\sigma^2_{\mathrm{f}} = \frac{\sigma^2}{N^2} \approx \begin{cases} 1/6 & \text{for } \delta < 1/N \\ 2/(N\delta)^2 & \text{for } \delta > 1/N \end{cases} \tag{A6}$$

Thus, the relative uncertainty in the response $\Delta\langle f\rangle$ is

$$\sigma_{\mathrm{rel}} = \frac{\sigma_{\mathrm{f}}}{\Delta\langle f\rangle} \approx \begin{cases} \sqrt{6}/(N\delta) & \text{for } \delta < 1/N \\ \sqrt{2}/(N\delta) & \text{for } \delta > 1/N \end{cases} \tag{A7}$$

As can be seen in Fig. 3, the two limits fairly well describe the behavior over the full space of signals and response. The sensitivity can be calculated from the average response from the definition in Eq. 2 of the main text. This gives

$$S_{\langle \mathrm{f}\rangle,\alpha} \approx \begin{cases} N/6 & \text{for } \delta < 1/N \\ 1/\delta & \text{for } \delta > 1/N \end{cases} \tag{A8}$$

The average time for the switch to move from one stationary state to the other can be calculated from the distance $\Delta\langle n\rangle$ that the average moves divided by the net rate of movement:

$$\tau = \frac{\Delta\langle n\rangle}{k_1 - k_2} = \frac{N\Delta\langle f\rangle}{k_2\delta} \tag{A9}$$

Here, $k_2 = k_{\mathrm{cat}}N_{\mathrm{E}}$ is determined by the catalytic rate of the enzyme and the number of demodification enzymes, N_{E}. Thus the switching time is determined by the ratio of targets to converters, $N/N_{\mathrm{E}} = C_0/C_{\mathrm{E}}$, and is independent of the size of the system for any given signal δ. For a given response, $\Delta\langle f\rangle = 0.8$, the required signal is $\delta^{80} \approx 10/N$ (Eq. 6), and the switching time is

$$\tau^{80} \approx \frac{0.8N}{10k_{\mathrm{cat}}}\frac{C_0}{C_{\mathrm{E}}} \approx \frac{S^{80}_{\langle \mathrm{f}\rangle,\alpha}C_0}{k_{\mathrm{cat}}C_{\mathrm{E}}} \tag{A10}$$

where in the last step the sensitivity from Eq. 7 has replaced the factor N. Numerical integration shows that the same relation holds also for the macroscopic switch (calculations not shown) independently of the value of K_{M}. Thus, switching time is directly proportional to the sensitivity. This is not unexpected, since a large sensitivity requires a small change in signal and a small change leads to a weaker force and a longer switching time. For a small change across the switch (when $\delta < 1/N$), using Eqs. A5 and A9, one finds

$$\tau = \frac{N^2}{6k_2} \tag{A11}$$

This corresponds to the diffusional relaxation time across the $N+1$ states of the scheme (Eq. 4). As it involves only a small shift, $\Delta\langle f\rangle < \frac{1}{6}$ from Eq. A5, in the switch, it is of less interest for the ultrasensitivity considered here. On the other hand, this limit shows that the simple calculation of the mean time as in Eq. A9 is correct even in the limit where the average does not move much and the main change is a rearrangement of the probability distribution across the whole sample space.

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