Ultrasensitivity in Biochemical Systems Controlled by Covalent Modification

INTERPLAY BETWEEN ZERO-ORDER AND MULTISTEP EFFECTS*

Albert Goldbeter‡ and Daniel E. Koshland, Jr.§
From the ‡Faculté des Sciences, Université Libre de Bruxelles, Campus Plaine C, P. 291, B-1050 Brussels, Belgium and the §Department of Biochemistry, University of California, Berkeley, California 94720

A previous analysis of covalent modification systems (Goldbeter, A., and Koshland, D. E., Jr. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6840–6844) showed that steep transitions in the amount of modified protein can occur when the converter enzymes are saturated by their protein substrate. This “zero-order ultrasensitivity” can further be amplified when an effector acts at more than one step in a monocylic or multicyclic cascade of covalent modification. We analyze the limitations of the latter “multistep ultrasensitivity” and show how it can combine with the zero-order effect to enhance the sensitivity of biochemical systems controlled by covalent modification.

In all biological systems, it is necessary to increase or decrease activities in response to external and internal signals. The sensitivity of the system to such signals becomes an important function of the regulatory controls. The terms “ultrasensitivity” and “subsensitivity” (1, 2) have been defined to indicate cases in which the sensitivity is greater or less than that to be expected from the standard hyperbolic (Michaelis-Menten) response.

Three potential sources of ultrasensitivity have been recognized: (a) the positive cooperativity of allosteric proteins, (b) the multistep effect when the same effector acts at more than one step in a pathway, and (c) the zero-order effect in covalent modification when at least one of the converter enzymes is saturated with its protein substrate.

Positive cooperativity was first recognized by Bohr in the case of hemoglobin (3) and is conveniently expressed quantitatively in terms of the Hill coefficient (4). A Hill coefficient as high as 3.8 has been shown in CTP synthetase but most enzymes have appreciably smaller Hill coefficients and many are subsensitive, i.e. negatively cooperative (5). The second potential source of ultrasensitivity is exemplified by the effective appearance of cAMP in 5 different steps of the glycogen cascade as revealed in the classic studies of Krebs, Fisher, Cohen and others (6, 7). How much sensitivity is generated by these multiple inputs has not been measured, but it is clearly well below a 5th power dependence on cyclic AMP concentration. Some quantitative aspects of multiple inputs have been developed by Chock and Stadtman (8) and applied to the glutamine synthetase cascade (9). The existence of multiple inputs does not automatically produce ultrasensitivity. The fact that the constants of the effectors must be in a narrow range to produce advantages for multiple-step and allosteric sensitivity has been discussed (1). The third source of ultrasensitivity, i.e. the zero-order effect, has been recently uncovered theoretically (2, 10) and demonstrated experimentally in the phosphorylation of isocitrate dehydrogenase (11).

In a highly regulated metabolic system more than one of these effects may be operating at the same time. It is important, therefore, to evaluate the limitations and potentials of each effect and their interplay. This is particularly important in the case of multistep and zero-order effects since both can (and have been observed in the isocitrate dehydrogenase case) occur together. In this paper, we shall explore the potentialities and limitations of zero-order and multistep effects and examine how their interplay enhances the sensitivity of a pathway to regulatory control.

Theory of Zero-Order and Multistep Effects

Properties of Zero-order Ultrasensitivity in Covalent Modification

In a covalent modification system in which (Equation 1) a protein $W$ is reversibly modified between $W_1$ and $W_2$ by converter enzymes $E_1$ and $E_2$, the steady-state fraction of modified protein, $W^*/W_t$, is given by Equation 2. For convenience we shall simply use the mole fraction $W^*$ to mean the ratio $(W^*/W_t)$. (See Refs. 2 and 10 for a detailed derivation of Equations 2–6, 10, and 12 below).

$$ W = \begin{cases} E_1 & \text{if } W^* \\ E_2 & \text{if } W^* \end{cases} \quad (1) $$

$$ \left(\frac{W^*}{W_t}\right) = \frac{\phi + 4K_4\left(V_i - 1\right)}{2\left(V_i^2 - 1\right)} \quad (2) $$

with

$$ \phi = \left(\frac{V_i - 1}{K_i}ight) - K_4 \left(\frac{V_i^2}{K_4^2 + V_i^2}\right) \quad (3) $$

Here, $V_i = k_iE_{1T}$ and $V_2 = k_2E_{2T}$ are the maximum rates of enzymes $E_1$ and $E_2$ at a given effector concentration (see "Properties of Multistep Ultrasensitivity in Covalent Modification"); $K_i = K_{ad}/W_t$ and $K_4 = K_{ad}/W_t$ are the Michaelis constants of $E_1$ and $E_2$ divided by the total amount of target protein.

Thus, when $V_i/V_2$ is increased from an initial low value, the fraction of modified protein progressively rises from a
value much smaller than unity to a value close to unity when $V_1$ exceeds $V_2$. The ratio of modification and demodification rates corresponding to a given value of $W^*$ is given by Equation 4. In particular, the midtransition point, where $W^* = W = 0.5$, corresponds to the ratio of modification rates shown in Equation 5.

$$V_1 = \frac{W^*(1 - W^* + K_m)}{V_m} (1 - W^*)(W^* + K_m)$$

where $V_m$ represents the maximum activity of the modifying enzymes and $K_m$ is the Michaelis constant. The latter equation indicates how the ratio of $V_1/V_2$ yielding half-maximum modification will be affected by changes in $K_m$ and $K_m$ with respect to $W_T$. We have previously shown that the transition $W \rightarrow W^*$ becomes steeper and steeper as $K_m$ and $K_m$ decrease below $W_T$. This phenomenon was termed "zero-order ultrasensitivity" (1, 2) to indicate that the enhancement in sensitivity with respect to a Michaelian response depends on at least one of the converter enzymes operating outside the first-order range.

The effect of altering the relative values of the Michaelis constants $K_{m1}$ and $K_{m2}$ in such a way that one is above and the other below $W_T$ is shown in Fig. 1. It can be seen that the top of the modification curve is steeper when $K_{m1} < K_{m2}$, whereas the converse is true when $K_{m1} > K_{m2}$. Then the completion of the $W \rightarrow W^*$ transition is very shallow as $V_1/V_2$ increases, whereas the initial part of the curve is steeper. In addition, as expected from Equation 5, the midpoint of the transition curve is shifted to higher values of $V_1/V_2$ when $K_{m1}$ increases with respect to $K_{m2}$.

It is important to evaluate the time dependence of the shift in the amount of modified protein, since regulation must be achieved in physiologically reasonable times. The time evolution of the modification system subsequent to a change in the ratio of $V_1/V_2$ is governed by the differential Equation 6.

$$\frac{dW^*}{dt} = \frac{V_1}{V_m} (V_1/V_2)(1 - W^*) - \frac{W^*}{K_m + W^*}$$

where $V_1$ and $V_2$ represent the maximum activities of the modifying enzymes $E_1$ and $E_2$, respectively, and $K_m$ and $K_m$ are the activation and inhibition constants.

In Fig. 2 is shown the variation of the fraction of modified protein ($W^*/W_T$) following instantaneous increases in the ratio $(V_1/V_2)$ when the modifying enzymes operate in the zero-order and the first-order kinetic domains. The increase in the ratio of modification rates is achieved here by an increase in $V_1$, for a fixed value of $V_2$. A comparison of $a$ and $b$ shows that in the former case a minor change in the ratio of modification rates (from 0.9 to 1.1) suffices to shift the target protein from 90% unmodified to 90% modified form, whereas a change by a factor of at least 50 is needed in the absence of zero-order effect. When the variation in $V_1/V_2$ is not particularly rapid, e.g. when $V_1/V_2$ rises to the final value with a half-time of the order of minutes, zero-order ultrasensitivity is associated with the existence of a time lag of similar duration. This time lag in the rise of modified protein (see Fig. 5 of Ref. 2) is due to the time required for $V_1/V_2$ to pass over the modification threshold, illustrated by Fig. 1 and Fig. 2a.

To alter $V_1$ or $V_2$, it is necessary to activate or inhibit enzymes $E_1$ and $E_2$. To exemplify the effect of the mode of control of these enzymes on the modification system, we shall consider both competitive and noncompetitive inhibition of $E_2$ by an effector $J$. The reaction rate in these conditions is respectively expressed in Equations 7 and 8.

$$V_1 = \frac{V_m W^*}{K_m (1 + J/K_m)} + W^*$$

$$V_2 = \frac{V_m W^*}{K_m (1 + J/K_m)}$$

where $V_{m1}$ and $K_{m2}$ represent the maximum rate and the Michaelis constant of $E_2$ in the absence of effector; $J$ represents the concentration of this effector; $K_{m2}$ is the inhibition constant. The effect of $J$ is to modify the maximum rate but not the Michaelis constant in noncompetitive inhibition, whereas the reverse obtains for competitive inhibition. Such a difference results in markedly different behavior in modification kinetics, as shown in Fig. 3. The modification curve appears to be steeper in the case of noncompetitive inhibition. Such an effect originates in part from the fact that in competitive inhibition, zero-order ultrasensitivity progressively diminishes as the kinetics of $E_2$ goes from zero-order into first-order (Equation 7).

Properties of Multistep Ultrasensitivity in Covalent Modification

Monocyclic System—To examine the multistep effect in the presence of complicated zero-order effects, we shall examine the influence of an effector $J$ when the enzymes $E_1$ and $E_2$ operate in their first-order region. It has previously been shown that zero-order ultrasensitivity is absent in this case (2).

A simple illustrative case occurs when $J$ is an absolute activator of $E_1$ and a noncompetitive inhibitor of $E_2$. In that case the ratio of maximum rates $V_1/V_2$ is given by Equation 9

$$\frac{V_1}{V_2} = \frac{V_{m1}}{V_{m2}} \left( \frac{J(K_m + J)}{K_m^2(K_m + J)} \right)$$

where $V_{m1}$ and $V_{m2}$ are the maximum activities of $E_1$ and $E_2$ in the presence and absence of $J$, respectively, and $K_m$ and $K_m$ are the activation and inhibition constants.

The variation of $W^*$ as a function of $V_1/V_2$ in the first-order region is of Michaelian form, as shown by Equation 10
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Time After Increase in \( \frac{V_1}{V_2} \) (seconds)

**Fig. 2.** Time evolution to steady state of the fraction of modified protein in the domains of zero-order (a) and first-order (b) kinetics. The ratios of modification rates \( V_1/V_2 \) are changed at time zero by increasing \( V_1 \), for example, by addition of an allosteric effector. The initial and final values of \( V_1/V_2 \) are shown on the curves for the intervals before and after addition of the effector. The curves are obtained by numerical integration of Equation 6 for \( W_T = 100 \mu M; a, K_m = K_m = 10^{-2} W_T, V_2 = 10 \mu M s^{-1}; b, K_m = K_m = 10 W_T \) and \( V_2 = 20 \mu M s^{-1} \). For an enzyme concentration \( EZ_T = 1 \mu M \), these values of \( V_2 \) correspond to turnover numbers of 10 or 20 s^{-1}, which compare to those observed experimentally. For example, the protein phosphatase I (1 \mu M) acts on phosphorylase \( \alpha \) (100 \mu M) with a \( k_{cat} = 3 s^{-1} \) (7). Phosphorylase kinase (4 \mu M) acts on glycogen phosphorylase (100 \mu M) with a \( k_{cat} = 158 s^{-1} \) (7).

\[
\frac{W^*}{W_T} = \frac{V_1/V_2}{K_1 + V_1/K_2 + V_2}
\]  

(see Ref. 1).

Taking into account Equations 9 and 10, one can compute the value of the response coefficient \( R_J \) (Equation 11) corresponding to the modification curve of \( W^* \) as a function of \( J \).

\[
R_J = \frac{9(\theta - 1) + [(\theta - 1)^2 + 36(\theta K_m/K_m)]^{1/2}}{[(\theta - 9) + [(\theta - 9)^2 + 36(\theta K_m/K_m)]^{1/2}}
\]  

(11)

where \( \theta = (K_m/K_m)/(V_m/V_m) \).

The response coefficient \( R_J \) has been previously defined as the value of controlling factor, \( J \), which produces 90% of the maximum response in proportion to one that gives a 10% response, i.e. \( J_{0.9}/J_{0.1} \), where \( J \) can be an allosteric effector, a substrate, or a stimulus, etc. The smaller the \( R_J \), the more sensitive is the response. Here, the response coefficient \( R_J \) given by Equation 11 corresponds to the 90 and 10% values of the interval from an initial value \( W^* \), to a final value \( W^* \), where the initial and final values are the levels of the modified protein when \( J \) goes from zero to infinity. The reason for this, as will be seen below, is that some of the modification curves reach a plateau while the target protein is not fully converted into \( W^* \). A similar flattening of saturation curves has been observed for allosteric interactions (12, 13).

A reciprocal measure of \( R_J \) has some advantages, since it increases numerically with increased sensitivity. For a Michaelian system, \( R_J = 81 (1, 2, 12) \). We have therefore plotted the reciprocal value \( 81/R_J \) which we call the "relative sensitivity coefficient," as a function of \((K_m/K_m)/(V_m/V_m)\) in Fig. 4, for different values of \( K_{m1} \) and \( K_{m2} \). As described in the figure legend, the relative sensitivity of this index reaches a maximum value of 9, i.e. the system is nine times more sensitive than a Michaelian system, when the effector acts in 2 steps, which is the theoretical maximum for a cooperative protein in which the Hill coefficient is 2.

Therefore, it is seen that the optimum multistep effect with \( J \) acting in two steps almost reaches the value of 9, which is logical since, from Equation 9, the optimum relationship between \( V_1/V_2 \) and the effector involves at most a proportion to \( J^2 \). This occurs when \( K_{m1}/K_{m2} \) is very large and \((K_m/K_m)/(V_m/V_m) \) = 1. There is actually quite a large plateau region when \( K_{m1}/K_{m2} \) is 10^2, which decreases as this ratio decreases. Thus, the lower the ratio of \( K_{m1}/K_{m2} \), the lower the sensitivity. Moreover, it is the ratio of \((K_m/K_m)/(V_m/V_m)\) not the absolute value of any individual parameter, which controls the sensitivity. The symmetry of the curves is logical since
Multistep Effect in a Bicyclic Cascade — It is next desirable to consider a multistep effect in a bicyclic cascade of the type shown in Fig. 5. The protein modified in the first cycle ($W^*$) catalyzes the $Z \rightarrow Z^*$ conversion in the second cycle. The reversal of that conversion is catalyzed by $E_3$. To determine the multistep effect alone we assume that all the enzymes operate in the first-order region. $J$ is assumed to be a noncompetitive inhibitor of $E_2$ and $E_3$. (An analogous situation of $E_2$ and $E_3$ inhibition occurs in glycogen metabolism where the phosphorylated inhibitor protein inhibits the phosphatase which acts on various phosphorylated proteins (14).)

In the specific case shown in Fig. 6, the inhibition constants are assumed to be equal, i.e. $K_{2a} = K_{2b} = 0.2$. Other parameters were selected to give a sizeable multistep effect. Curves $c$ and $d$ show the effect of inhibition of $E_2$ alone or $E_3$ alone. Curve $b$ is the Michaelian reference curve. The combination of two inhibitions is shown in curve $a$ and gives a response coefficient $R_J$ of 11.6 (or a relative sensitivity of 7). This closely corresponds to the relative sensitivity of a fully cooperative dimeric enzyme.

If $J$ acts only in the first cycle of the bicyclic cascade but on two enzymes (activating $E_1$ and inhibiting $E_3$) the change in $Z^*$ is smaller per change in $J$ than the change in $W^*$. The second cycle in that case diminishes the sensitivity of the first cycle.

It is also seen that inhibition of $E_3$ is more effective than inhibition of $E_2$ in relation to the sensitivity of changes in $Z^*$ per change in $J$. This can be explained qualitatively by the following reasoning. If $E_3$ is fully inhibited, all $Z$ goes to $Z^*$ as the ratio of modification rates goes to infinity. In contrast, if $E_2$ is fully inhibited, all $W$ goes to $W^*$, but this does not cause necessarily all $Z$ to go to $Z^*$, since $E_3$ is active. Hence, a controlled cycle followed by a cycle which has no effector control will be less sensitive than a single cycle or a bicyclic cascade in which the last cycle is regulated.

Interplay between the Zero-order and Multistep Effects

Monocyclic System — When an effector acts on two converter enzymes in a system of the type shown in Equation 1, ultrasensitivity could result from a multistep effect, a zero-order effect, or a combination of the two. In such a system the fraction of the substrate protein in the modified form $W^*$ is given by Equation 2 and it is possible to factor out the relative contributions of the multistep and zero-order effects by considering two situations. When $K_1$ and $K_2$ are both much larger than unity, i.e. when both enzymes are operating in the first order region, it has been established that there is no zero-order effect (2). Thus, a calculation using a low value of $(W^*/W)$ which satisfies a first-order situation should be able to separate the multistep effect from the zero-order effect. This is shown for an illustrative case in Fig. 7, where curves $b$ and $c$ show the zero-order and multistep effects alone, and curve $a$ shows the combination of the two effects.

To evaluate the zero-order effect alone we have used the
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Fig. 7. Interplay between zero-order and multistep ultrasensitivity in the monocyclic system of Equation 1. Curve b illustrates the multistep effect alone, with \( (V_{m1}/V_{m2}) = 1 \), \( (K_{m1}/K_{m2}) = 0.1 \), and \( (K_{m1}/K_{m2}) = 100 \). These conditions favor multistep ultrasensitivity (see Fig. 3). The curve is obtained according to Equations 9 and 10. Curve c shows the zero-order effect alone. The curve is identical to curve b in Fig. 1. Curve a results from the combination of multistep and zero-order effects. It is obtained from Equations 2 and 9 for the same parameter values as for curve b, with \( K_{m1} = K_{m2} = 0.1 W_t \).

Table I

| Reduced Michaelis constants of \( E_1 \) and \( E_2 \) | Multistep and zero-order ultrasensitivityalone | Multistep and zero-order ultrasensitivity cumulated |
|--------------------------------------------------|-------------------------------------------|-----------------------------------------------|
| \( K_s = (K_{m1}/W_t) \)                         | \( R_t \)                                | \( R_t \)                                      |
|                                                  | \( 81 \)                                | \( 81 \)                                      |
|                                                  | \( 81 \)                                | \( 81 \)                                      |
| \( K_s = (K_{m1}/W_t) \)                         | \( R_t \)                                | \( R_t \)                                      |
|                                                  | \( 81 \)                                | \( 81 \)                                      |
|                                                  | \( 81 \)                                | \( 81 \)                                      |
| \( K_s = (K_{m1}/W_t) \)                         | \( R_t \)                                | \( R_t \)                                      |
|                                                  | \( 81 \)                                | \( 81 \)                                      |
|                                                  | \( 81 \)                                | \( 81 \)                                      |

Equation 12 obtained in the derivation of the zero-order effect (2).

\[
R_t = \frac{81(K_{m1} + 0.1 W_t)(K_{m1} + 0.1 W_t)}{(K_{m1} + 0.9 W_t)(K_{m1} + 0.9 W_t)}
\]

This equation gives the quantitative relationship for zero-order steepness of the curve regardless of the means of influencing \( V_1 \) or \( V_2 \). \( R_t \) represents the ratio of \( (V_t/V_2) \) at 90% of the maximum response versus \( (V_t/V_2) \) at 10% of the maximum response. The advantage of this formulation is that the effect of changing \( V_t/V_2 \) is separated from the method of achieving that change. It is seen from the example of Fig. 7 that most of the ultrasensitivity in that case comes from the zero-order effect but there is a slight additional sensitivity from the multistep effect.

Both effects can be cumulative but they are not simply multiplicative. To evaluate the relative contributions under various circumstances, we have shown in Table I the relative sensitivity indices for the multistep effect alone (column 1), the zero order effect alone (column 2), and the combined effects (column 3) for various values of \( K_{m1} \) and \( K_{m2} \).

At large values of \( K_{m1} \) and \( K_{m2} \) (\( K_{m1} = K_{m2} = 100 W_t \)), there is no zero-order effect, and the multistep effect for this particular combination of constants for the cycle yields a \( R_t \) value of 11.7. As \( K_{m1} \) and \( K_{m2} \) decrease, the multistep effect should not change since the only parameter being varied is the concentration of the substrate \( W_t \). This is seen to be true and the zero-order effect becomes more and more significant until a \( R_t \) value of 1.2 is reached at \( K_{m1} = K_{m2} = 10^{-5} W_t \). The \( R_t \) value associated with the cumulated zero-order and multistep effects is then close to 1.1. The minimum possible value for \( R_t \) is 1, so this is an extraordinarily high ultrasensitivity, corresponding to an allosteric protein with a Hill coefficient of 50. It can be seen from this illustrative example that there is not a simple multiplicative relationship between the two effects but the "global" ultrasensitivity can indeed be greater than either multistep or zero order alone.

How to evaluate the contribution of each effect experimentally becomes apparent from the mathematical relationships described above. One can simply study the kinetics at a value of \( W_t \) sufficiently low so that only the multistep effect is observed. Since the parameters of the converter enzymes should not change as a function of the target protein concentration, the relative contribution of multistep ultrasensitivity can be readily ascertained. To determine the contributions of zero-order ultrasensitivity alone, one can calculate the ratio \( R_t \) described in Equation 12. The value of \( R_t \) can be compared with the overall response as shown in Table I to determine the relative contributions of each effect. This procedure was followed in analyzing the phosphorylation of isocitrate dehydrogenase (11).

The multistep effect will frequently be subsensitive \( (R_t > 81) \) but the overall response will be ultrasensitive because of the influence of the zero-order effect. Such a situation is illustrated in Fig. 8 where the transition curve for \( W^* \) as a function of the effector \( J \) shows an intermediary plateau region. In this case the multistep effect alone is less sensitive than a Michaelis-Menten curve. However, combining such a multistep effect with a zero-order ultrasensitivity equivalent to that of curve c in Fig. 7 gives a response close to a Michaelis-Menten curve.

This situation is probably a fairly representative one. Because of the stringent demands on constants for multistep effects, this effect will only rarely be ultrasensitive. On the other hand, activating one step and inhibiting the reverse step is an excellent way to alter \( V_1/V_2 \) and thus implement a zero-order effect. The combination of these two phenomena should therefore produce increased sensitivity in many biological systems. As an example we can consider the situation...
illustrated in Table I for \( K_m = K_{m2} = 10^{-1} \) W. The values of \( V_{V1} \) at 10 and 90% \( W^* \) give an \( R_T \) of 3.24 which relates to the zero-order effect alone. The multistep effect can be calculated from Table I by assuming that \( W_T \) is very small, which results in an \( R_T \) of 11.7 for the multistep effect alone. The combination of the two effects gives a combined \( R_T \) of 1.9, a highly ultrasensitive response equivalent to an allosteric protein with a Hill coefficient of 8.

**Bicyclic Cascade**—We return to the cascade considered in Fig. 5 and determine now the effect of zero-order ultrasensitivity when superimposed on the multistep effect. To allow comparison with curve a of Fig. 6, we take the same parameter values and consider the case where the Michaelis constants of the four enzymes of the cascade are small, compared to the total amounts \( W_T \) and \( Z_T \) of their protein substrates.

Fig. 9 shows that when the zero-order ultrasensitivity obtains in cycle 1 alone (a), in cycle 2 alone (b), or in both cycles (c), the transition in \( Z^* \) is sharper than with the multistep effect alone (curve a of Fig. 6). The latter is characterized by a \( R_T \) value of 13.5, whereas \( R_T \) drops to 6.4 in a, 2 in b, and 1.5 in c. These values are obtained for a case where the zero-order effect is not exceedingly favored, given that \( W_T \) and \( Z_T \) are only taken ten times larger than their related Michaelis constants (except \( K_{m2} \) which is equal to \( W_T \)). Increased sensitivity could be obtained with smaller values of the Michaelis constants.

An intriguing effect can be obtained as a result of the interplay between zero-order ultrasensitivity and multistep inputs of the effector when the inhibition constant of \( J \) for \( E_3 \) is well below its inhibition constant for \( E_5 \). If constants are selected so that full transformation of \( W \) into the active metabolite. Since it is obvious that the sharper the control the wider the range of control over a larger range.

**DISCUSSION**

The previous analyses of covalent modification systems have indicated that both the multistep effect and a zero-order effect can produce ultrasensitivity using parameters that are in the physiological range. Both are distinctly different from allosteric cooperativity but can combine with allosteric cooperativity and with each other to produce enhanced ultrasensitivity effects.

Multistep ultrasensitivity can arise from a single effector operating on different steps in a cascade. It has a theoretical limit based on the number of steps that the effector controls but it can approach close to that limit; for example, a single effector acting at two steps of the cascade will approach the ultrasensitivity of an allosteric dimer. If all the parameters are in the optimal range. The conditions for optimal sensitivity are numerous, however, and if these parameters (the inhibition constants, the maximum enzyme activities, the Michaelis constants, etc.) are not optimal, the ultrasensitivity will be decreased dramatically and the response may in some cases be appreciably subsensitive. One of the conditions in which the multi-step effect is optimized occurs when the constant for activation of the forward step, for example, a kinase, is

![Fig. 9. Interplay between zero-order and multistep ultrasensitivity in the bicyclic modification cascade of Fig. 5. The multistep effect of Fig. 6 (a) is associated with zero-order ultrasensitivity in cycle 1 alone (a), in cycle 2 alone (b), or in both cycles 1 and 2 (c). Curves are obtained by using equations similar to Equations 2 or 10 for \( W^*/W_T \) and \( Z^*/Z_T \), depending on whether zero-order ultrasensitivity is considered to occur or not. Parameter values are \( V_{V1}/V_{V2} = 1 \), \( K_{m2} = 2.10^{-8} \) M, \( K_{m2}/K_{m1} = 10 \), and \( K_{m2}/K_{m3} = 1 \), for all curves, with \( K_{m1} = 0.1 \) W_T and \( K_{m2} = 0.1 \) Z_T in the presence of a zero-order effect.](image-url)
much greater than the constant for inhibition of the reverse step, for example, a phosphatase. However, the reverse is not true, and only subsensitivity can be obtained when the dissociation constant for the activation step is far below the dissociation constant for the inhibition step. In principle, therefore, multistep ultrasensitivity can increase without limit if the number of steps controlled by the effector increases, but in practice it will be very unlikely if all the parameters can be in the optimal range.

Zero-order ultrasensitivity can therefore be far more effective than multistep ultrasensitivity, since it is possible to have an extremely dramatic step increase in activity in a single cycle. To obtain such a sizeable increase, however, again requires the values of all of the constants to be optimally related; moreover, the enzymes which are acted on must be saturating their converter enzymes. Obviously, in a multicycle cascade, this would put increasing demands on the concentration of enzymes. On the other hand, one does not need more than one or two cascades with appropriate constant to convert essentially 99% of an inactive enzyme to 99% in the active form. Zero-order ultrasensitivity therefore provides an effective switch mechanism which, because of the wide occurrence of protein covalent modification, could play a role in the control of a large number of cellular processes, both in normal and in pathological conditions.

In a multicycle cascade, the effector is not equally effective in any step. Thus, in a bicyclic cascade, for multistep control alone, it is far more efficient to exert regulation at the second of the two cycles. If the parameters for the second cycle are not optimal, the second cycle can severely dampen any ultrasensitivity generated in the first cycle.

An analysis of the interplay between the zero-order effect and the multistep effect shows that these two effects can act synergistically. In all cases this interplay tends to enhance the sensitivity gained for either effect alone. Zero-order ultrasensitivity tends to be the stronger of the two effects. When the multistep effect is subsensitive, the zero-order effect can even change the system to be at least as sensitive as a Michaelian system, and in some cases to become ultrasensitive. Either effect can of course give a further contribution to ultrasensitivity when the control of the converter enzymes, instead of being Michaelian, possesses some degree of cooperativity. Such is the case for the control of protein kinase by cyclic AMP (15).

The analysis suggests that zero-order ultrasensitivity can be distinguished from the multistep effect in any particular system simply by diluting the target protein from well above to well below the $K_a$ of the modifying enzymes. When the converter enzymes are operating in the first-order region, there will be no zero-order effect and the only contribution will come from the multistep effect. The calculations show that increasing the enzyme concentration when keeping all other parameters constant should, under most circumstances, reveal the extent of the zero-order ultrasensitivity alone.

We have examined the time evolution of the modification system under first-order and zero-order conditions to make sure that the system can shift to a new steady state in times that are physiologically reasonable. The results clearly show that quite rapid times can be obtained using values which are consistent with known kinase and phosphatase turnover numbers existing in the literature.

An interesting effect pertains to the existence of a plateau region in the modification curve when an effector controls more than one cycle in a cascade. Then, as the effector $J$ is varied, one can have two regions of zero-order ultrasensitivity separated by an intermediate region in which the system is practically insensitive to regulation by the effector $J$. The global sensitivity in going from 10 to 90% activity is therefore very low, but the steepness of the rise from 1 to 15% can be very abrupt, followed by a long plateau region and then resuming by a second abrupt rise in activity in a highly sensitive manner at some increased value of effector concentration. Whether this staircase or quantized effect exists in actual systems controlled by covalent modifications remains to be seen but it could be biologically effective, and previous predictions of such unusual plateau regions by similar theoretical studies for allosteric proteins (12, 13) turned out to have physiological counterparts. The existence of step transitions due to the zero-order effect means increased sensitivity but also a reduced range of control for a given effector. The staircase effect could provide both ultrasensitivity in response and the possibility of an extended range of control.

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