Solute Modulation of Conformational Equilibria in Intrinsic Membrane Proteins: Apparent "Cooperativity" without Binding

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ABSTRACT The activity of many membrane proteins depends on a conformational transition that is often strongly influenced by small membrane-soluble solutes. This allosteric modulation may be direct, involving binding to the protein at localized sites of varying specificity, or may be indirect, resulting from altered membrane properties. In the present paper, a general expression for solute-protein titration curves is predicted, using an indirect mechanism that couples solute-induced changes in the lateral pressure profile of the bilayer to a shift in protein conformational equilibrium. When the common practice of fitting dose-response data to the Hill equation is applied to these curves, the fits are found to be reasonably good, with large Hill coefficients. Because this would commonly be interpreted as evidence of the existence of multiple sites with strong positive cooperativity, it is argued that caution must therefore be exercised in the interpretation of titration data in the absence of direct evidence of the existence of binding sites. The form of the titration curve predicted from this lateral pressure mechanism is shown to be quite general for indirect mechanisms. It is also shown that this form is the same as would be obtained from classical models of binding cooperativity, such as that of Monod, Wyman, and Changeux, in the limit of an infinite number of sites with vanishingly small site affinity.

INTRODUCTION

The activity of intrinsic membrane proteins is often strongly yet reversibly influenced by the presence of small membrane-soluble (hydrophobic or amphiphilic) solutes. This allosteric modulation is often reflected in altered dose-response curves: the sensitivity of protein activity to its principal effector (agonist or substrate concentration, membrane voltage, light, etc.) varies with the aqueous concentration of the allosteric solute. The mechanisms by which this influence might be exerted can be divided into two classes. In the classical paradigm, the solute interacts directly by binding reversibly to sites on the protein, in which case the solute acts as a ligand. The "specificity," i.e., the strength, range, and localization of this binding, can vary widely (Eckenhoff and Johansson, 1997). Specific binding indicating relatively strong, more localized interactions might involve hydrogen bonds or Coulombic attractions, while nonspecific binding implies weaker and less localized effects that might arise from hydrophobic interactions or dispersion forces. If the relation between allosteric ligand binding and protein activity is readily measured, changes in the effector dose-response curve can then be interpreted in terms of microscopic binding characteristics.

In a second class of mechanisms, solutes modulate protein activity indirectly, i.e., without binding to the protein, even weakly. Consider as a common example an intrinsic membrane protein whose function depends on a conformational transition. Solubilization of a small hydrophobic or amphiphilic molecule can alter the thermodynamic and structural properties of the bilayer, which can shift the protein conformational equilibrium and thus modulate protein activity. Hydrophobic thickness, the distribution of lateral stresses and resulting curvature elastic properties, dipole potential, fluidity, and phase coexistence behavior (proximity to phase transitions and degree of microheterogeneity) all vary with membrane lipid/solute composition and have thus been suggested (Brown, 1997; deKruijff, 1997; Epand, 1996; Gruner, 1991; Hui, 1997; Lundbaek and Andersen, 1999; Morein et al., 1996; Mouritsen and Jørgensen, 1997; Mouritsen and Bloom, 1993; Nielsen et al., 1998; North and Cafiso, 1997) as having a potentially strong influence on membrane protein function. However, with regard to the influence of anesthetics on ion channel proteins, it has been noted (Franks and Lieb, 1994) that the changes in most of these properties (thickness, order parameter profiles, phase transition temperatures) are very small at the concentrations of anesthetics at which protein activity is known to be affected and can be produced in the absence of solute through slight changes in other variables such as temperature. Were there no bilayer properties that are both sensitive to incorporation of solutes and capable of influencing protein equilibria, it could be concluded that such indirect mechanisms are likely to play at most a minor role in the modulation of protein activity. However, it has been suggested (Gruner, 1991; Seddon and Templer, 1995; Cantor, 1997a, 1999) that the distribution of lateral stresses in bilayers may be such a property, because it is predicted (Cantor, 1997b, 1999) to be strongly affected by the incorporation of interfacially active solutes as well as by altered lipid composition (but not by small changes in temperature), and more importantly, it is mechanistically linked to altered protein conformational equilibria, as well as other protein or

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peptide equilibria, such as aggregation and membrane insertion.

Clearly, there are proteins for which experimental evidence has unambiguously identified the number and location of specific binding sites of membrane-soluble allosteric inhibitors or activators. However, in many systems direct evidence of such binding is lacking. In the absence of such evidence, is it possible to distinguish between solutes that act as ligands (perhaps weakly and diffusely) and those that influence a protein indirectly? To address this question it is useful to compare the mathematical forms of the titration (dose-response) curves that would be predicted by appropriate models for each of the two modes of solute influence on protein function: direct binding and indirect interactions. First a brief summary is presented of an indirect mechanism in which a redistribution of the lateral pressures that accompanies the addition of small concentrations of solutes to the bilayer alters the equilibrium between protein conformational states, from which a simple expression for the titration curve is obtained. It is then shown that this expression is quite general for a wide range of properties that might mediate indirect solute-protein interactions. These curves are then compared to the standard "logistic" equation (Hill, 1910) to which titration data are often fit. The quality of the fits and the large values of the Hill slope reveal that, in the absence of direct experimental evidence of the existence of solute binding sites, experimental dose-response curves arising from an indirect mechanism might easily be misinterpreted as cooperative binding to multiple sites. Finally, the titration curve predicted from the indirect mechanism is compared to the well-known predictions of ligand binding cooperativity in the framework of the MWC model (Monod et al., 1965; Wyman and Gill, 1990), in which the cooperativity derives from preferential binding of the solute to one of the protein conformational states. It is demonstrated that the indirect approach yields a dose-response relation that is mathematically identical to what would be predicted for classical binding models in the limit of an infinite number of infinitely weakly binding sites.

THEORY

An indirect mechanism: the lateral pressure profile

In recent work (Cantor, 1997a,b, 1999) a simple thermodynamic argument was combined with lattice statistical thermodynamic calculations to predict the effect of a redistribution of lateral stresses arising from a small change in bilayer composition on the conformational equilibria of proteins for which the change in cross-sectional area that accompanies the transition is nonuniform. A brief summary of the argument follows. An intrinsic protein is assumed to exist in one of two conformational states, t (inactive) or r (active), the activation of which is thus associated with the conformational transition $t \rightarrow r$. The cross-sectional areas of the protein in the transmembrane domain for each of the two states, $A_t(z)$ and $A_r(z)$, will generally vary with depth within the bilayer (z). The change in cross-sectional area that accompanies the transition is thus given by $\Delta A(z) = A_r(z) - A_t(z)$. The bilayer is characterized by a depth-dependent lateral pressure density $p(z)$ that depends on composition. A change in composition, such as the incorporation of a small solute, will result in a significant redistribution of the lateral pressures. Define the "standard state" of the bilayer to be the absence of added solute, with pressure profile $p_0(z)$. The change in the pressure profile with the addition of solute is then $\Delta p(z) = p(z) - p_0(z)$. Let $[t]_0$ and $[r]_0$ be the concentrations of the protein conformational states in the absence of solute (i.e., in a bilayer in its standard state), with conformational equilibrium $K = [r]_0/[t]_0$; the fraction of active protein is thus $F_0 = 1/(1 + K^{-1})$. In the presence of solute, the concentrations are denoted [t] and [r], and the fraction of active protein is denoted by F. The relationship between F and F_0 is obtained by equating the chemical potentials of the two states of the protein first in the absence and then in the presence of solutes. To a good approximation the dependence of the chemical potential of the protein in each conformational state on its own concentration and on the pressure distribution has a simple form (Cantor, 1997a); for the active (r) conformation,

$$\mu_r/RT = \mu_r^\circ/RT + \ln[r] + (k_BT)^{-1}\int p(z)\, A_r(z)dz, \quad (1)$$

where R is the gas constant, k_B is Boltzmann's constant, and T is the absolute temperature; an analogous expression obtains for the inactive (t) state. At equilibrium, $\Delta\mu = \mu_r - \mu_t = 0$, so

$$0 = \Delta\mu^\circ/RT + \ln [r]/[t] + (k_BT)^{-1}\int p(z)\Delta A(z)dz. \quad (2)$$

This equality must hold in the bilayer standard state (without solute) with pressure profile $p_0(z)$:

$$0 = \Delta\mu^\circ/RT + \ln[r]_0/[t]_0 + (k_BT)^{-1}\int p_0(z)\Delta A(z)dz. \quad (3)$$

Taking the difference of these two equations gives

$$[r]/[t] = Ke^{-\alpha}, \quad (4)$$

where $\alpha = (k_BT)^{-1}\int\Delta p(z)\Delta A(z)dz$. The fraction of protein in the active conformation is thus

$$F = 1/(1 + K^{-1}e^{\alpha}). \quad (5)$$

For the addition of a solute at concentration x, the effect on the pressure profile will be approximately linear in x at low membrane concentrations, so we can define $\beta = \alpha/x$, which is independent of x. (For small interfacially active solutes, lattice statistical mechanical calculations (Cantor, 1999, and

unpublished results) usually predict the linear regime to extend to quite high solute concentrations—certainly in excess of 10 mol%—and often the deviations are predicted to remain small at substantially higher concentrations, with the details depending on the solute and the bilayer lipids.) The sign of β determines whether the addition of solute increases or decreases the fraction of active protein, i.e., whether protein activity is enhanced ($\beta < 0$; $F_0 < F < 1$) or inhibited ($\beta > 0$; $F_0 > F > 0$). (Typically, for inhibition $K^{-1} \ll 1$, while for activation $K^{-1} \gg 1$.) It is common to express the fraction of active protein relative to its maximum value F_{max}. For inhibition, this occurs in the absence of solute ($x = 0$), and thus $F_{max} = F_0$, while for activation it occurs in the limit of large α, at which $F_{max} = 1$. For inhibition, the relative fraction of active protein is thus given by

$$f = F/F_{max} = (1 + K^{-1})/(1 + K^{-1}e^{\beta x}), \tag{6a}$$

while for activation,

$$f = F/F_{max} = 1/(1 + K^{-1}e^{\beta x}). \tag{6b}$$

These titration curves depend on two parameters, K and β. Unlike β, K is independent of the identity of the added solute. Titration data are usually plotted as f against $\log_{10}(x)$, so in Fig. 1 the predicted curves are plotted in this form for a range of values of K. Because β functions as a multiplicative scaling factor of x, a change in β corresponds to a horizontal shift of the curve, while the steepness of the curve is dictated by K. In the context of ligand binding, a horizontal shift is usually interpreted as a measure of binding affinity, and the steepness of the curve in terms of the degree of "cooperativity." Because variations in both of these apparent binding characteristics can easily be mimicked in this indirect mechanism, the potential for misinterpretation of an indirect solute-protein interaction as cooperative binding is evident.

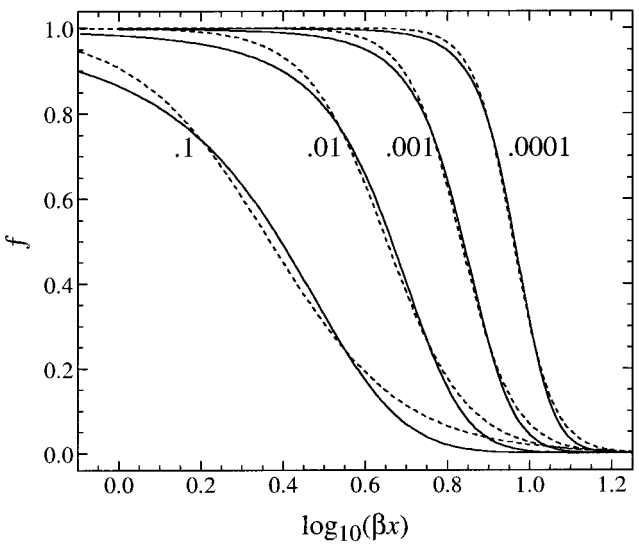

FIGURE 1 Predicted titration curves for allosteric inhibition ($\beta > 0$). f, the ratio of the fraction of protein in active form in the presence of solute to the fraction in the absence of solute, is plotted as a logarithmic function of solute concentration, with values of K^{-1} as indicated. Predictions from the indirect model (Eq. 6a, *solid curves*) are compared with best fits of the Hill equation $f = 1/[1 + (k_H x)^n]$ with respect to its parameters k_H and n (*dashed curves*). The best-fit values of n are essentially identical to the values of n_{inh} given as an explicit function of K in Eq. 12.

The exponential dependence on x in Eq. 5 arises from the fact that the chemical potential of the protein varies logarithmically with its own concentration but linearly with a pressure redistribution, which in turn depends linearly on (low) solute concentration. This result is thus expected to be quite general; any membrane property on which the difference in protein chemical potentials depends linearly will yield the same form for F as in Eq. 5. An example of such a property that has been considered in considerable detail (Mouritsen and Bloom, 1993; Nielsen et al., 1998) is the mismatch between the hydrophobic thickness of the bilayer h and that of the protein ξ. For the present purpose of deriving the form of the titration equation, a crude approximation much simpler than that of Nielsen et al. (1998) can be used in which this mismatch is described through an additive free energy contribution that depends only on the magnitude of the area of hydrophobic mismatch $c|(\xi - h)|$, where c represents the circumference of a protein cross-sectional slice in the mismatch region. In general, both c and ξ may depend on the protein conformational state. Incorporation of solute into a bilayer of thickness h_0 will change the bilayer thickness by an amount $\delta h = h - h_0$ that (for low concentrations of solute) will vary in proportion to solute concentration: $\delta h \approx \lambda x$. The chemical potential of each protein state takes a simple form: for the active conformation,

$$\mu_r/RT = \mu_r^\circ/RT + \ln[r] + \epsilon c_r|(\xi_r - h)|, \tag{7}$$

where ϵ represents the free energy (in units of RT) per unit area of mismatch; an analogous expression obtains for the inactive state. Four cases are possible: I ($\xi_r > h$, $\xi_t > h$); II ($\xi_r > h$, $\xi_t < h$); III ($\xi_r < h$, $\xi_t > h$); IV ($\xi_r < h$, $\xi_t < h$). For case I, setting the chemical potentials equal gives

$$\ln[r]/[t] = -\Delta\mu^\circ/RT - \epsilon[\Delta(c\xi) - h\Delta c], \tag{8}$$

where $\Delta c = c_r - c_t$. In the absence of solute, Eq. 8 becomes

$$\ln[r]_0/[t]_0 = -\Delta\mu^\circ/RT - \epsilon[\Delta(c\xi) - h_0\Delta c]. \tag{9}$$

Eliminating the difference in standard chemical potentials, $\Delta\mu^\circ$, from Eqs. 8 and 9 gives

$$[r]/[t] = Ke^{-\beta x}, \tag{10}$$

which is identical to Eq. 4, with $\beta = \epsilon\lambda\Delta c$; note that β can be of either sign, depending on the sign of λ (i.e., on whether the bilayer thickens or thins with added solute). For the remaining cases, the equilibrium still depends exponentially on solute concentration, but with different coefficients: $\beta = \epsilon\lambda(c_r + c_t)$, $-\epsilon\lambda(c_r + c_t)$, and $-\epsilon\lambda\Delta c$, for cases II, III, and IV, respectively.

Direct allosteric mechanisms: ligand binding

The isotherm for binding of a ligand to a protein with a single site is described in terms of the degree of saturation $f = kx/(1 + kx)$, which has only one parameter, the binding constant k. For proteins with multiple binding sites, analysis and interpretation of the binding curves can become complicated, particularly if the sites are inequivalent or if the binding is cooperative, i.e., when binding of a ligand to one site influences the intrinsic affinity of ligands to other sites. The relationship between protein activity and the distribution of proteins with varying degrees of ligation may also be complex. Still, the fundamental statistical thermodynamic principles underlying cooperativity are well understood (Hill, 1984; Wyman and Gill, 1990; DiCera, 1995). Various models of cooperativity (Monod et al., 1965; Koshland et al., 1966) have been developed that provide analytical expressions for the dependence of the fraction of bound sites on ligand concentration. Experimental measurements of binding titration curves can be fit to model predictions using only one or two adjustable parameters, the values of which provide estimates of the degree of cooperativity, but only if the number of binding sites is established independently. In many cases, the experimental data do fall reasonably well on the curves predicted by such models. Of course, the resulting interpretation has significance only if the model is a reasonably accurate representation of the actual mechanism of solute-protein interaction. In particular, if different microscopic models predict similar binding curves, than a titration experiment alone obviously cannot be used to distinguish among the models.

Hill plots

The well-known equation of A. V. Hill (1910) is often used to fit and interpret experimental titration data in terms of binding cooperativity; it should thus be examined closely and compared to the predictions of indirect mechanisms. It is not derived from any model of cooperativity; no change in protein conformational state is presumed. The approach simply assumes that the cooperativity among the n equivalent sites on the protein (regardless of its origins) is so strong that the protein only exists in one of two ligation states, all sites empty or all sites bound; i.e., the probability of finding a protein with some but not all of the sites bound is negligibly small. The resulting equation for y, the fraction of protein with bound sites, is given by

$$y = \frac{(k_H x)^n}{1 + (k_H x)^n}. \qquad (11)$$

Obviously, allosteric inhibition occurs if the protein is less active with than without bound ligand; activation results from the reverse. In the limit that the protein is assumed to be completely inactive in one ligation state, protein activity (relative to its maximum activity) is given either by $f = 1 - y$ (inhibition) or by $f = y$ (activation).

To the degree that ligand binding is not infinitely cooperative, Eq. 11 loses validity and would thus be expected to describe experimental data less well. For example, the slope of a plot of $\ln[y/(1 - y)]$ against $\ln(x)$ typically goes through a maximum at intermediate ligand concentration and approaches 1 in the limits $f \to 0$ and $f \to 1$, whereas if Eq. 11 were valid, such a plot would give a straight line with slope n. Nonetheless, Eq. 11 is frequently used to fit experimental data, with the interpretation that a good fit provides evidence for the existence of binding, and that the Hill coefficient (the magnitude of the derivative of $f/(1 - f)$ with respect to $\ln(x)$ evaluated at $f = 0.5$) provides a useful measure of the degree of cooperativity.

The danger of such interpretations is clearly demonstrated in Fig. 1, in which predictions of the indirect model of solute inhibition (Eq. 6a) are plotted along with best fits of the Hill equation for inhibition $f = 1 - y$ with respect to its two adjustable parameters, k_H and n. Curves are presented over a range of values of $K^{-1} \ll 1$. Clearly, the fits are quite good, particularly for small K^{-1}. (For allosteric activation, an analogous set of inverted curves obtains, with a similar quality of fits.) Using Eq. 6a, it is readily shown that at $f = 0.5$, $\alpha = \ln(K + 2)$, and thus the indirect model for inhibition predicts a Hill coefficient:

$$n_{inh} = [(1 + K^{-1})/(1 + 2K^{-1})] \ln(K + 2). \qquad (12)$$

[For activation (Eq. 6 *b*, $K \ll 1$, $\beta < 0$), the Hill coefficient takes a simpler form, $n_{act} = -\ln K$]. For small K^{-1}, $n_{inh} \approx \ln K > 1$; a multiplicative decrease in K^{-1} corresponds to an additive increase in n_{inh}. (The values of n obtained from fits of the entire curve are almost identical to the values of n_{inh} given by Eq. 12.) Clearly, the combination of the good quality of the fit and the existence of a large Hill coefficient could easily be misinterpreted as evidence not only for binding to multiple sites, but for the existence of strong cooperativity.

Relation between indirect mechanisms and the MWC model

Many approximate descriptions of ligand binding have been proposed in which cooperativity arises from a coupling of ligand binding to a conformational transition of a protein comprising multiple subunits. Such descriptions seem particularly relevant, given the considerable evidence that the function of many membrane proteins is associated with conformational changes. A canonical example is the model developed by Monod, Wyman, and Changeux (Monod et al., 1965; Wyman and Gill, 1990). In this (MWC) approach, the protein is assumed to be an oligomer of n identical subunits. Each subunit has one ligand-binding site and is assumed to exist in one of two conformational states, t (inactive) and r (active), using the same notation as in the previous section. The cooperativity arises from the assumption that the site binding affinity differs for the two protein states. It is further assumed that the transition is concerted,

i.e., all of the subunits of the protein must be in the same conformational state. With these approximations, the fraction of protein in the active conformation with the addition of ligand is given by

$$F = \left[1 + K^{-1}\left(\frac{1 + k_t x}{1 + k_r x}\right)^n\right]^{-1} \quad (13)$$

where k_t and k_r are the microscopic binding constants of the ligand to the subunit site in the inactive and active configurations, respectively. The greatest cooperativity occurs when k_t and k_r are very different, either $k_t \gg k_r$ (inhibition) or $k_r \gg k_t$ (activation). For these two extreme cases, Eq. 13 can be reexpressed relative to the maximum value F_{max} as

$$f = F/F_{max} = \frac{1 + K^{-1}}{1 + K^{-1}(1 + k_t x)^n} \quad \text{(inhibition)} \quad (14a)$$

$$f = F/F_{max} = \frac{1}{1 + K^{-1}(1 + k_r x)^{-n}} \quad \text{(activation)} \quad (14b)$$

where $F_{max} = F_0$ for inhibition and $F_{max} = 1$ for activation.

Both the indirect mechanism and the MWC binding model are based on the influence of the solute on protein conformational equilibria, so it might be expected that the form of the resulting titration curves might well be related. In fact, it is straightforward to show that the indirect model obtains from the MWC model in the limit of vanishingly small "specificity," i.e., by increasing the number of sites to infinity while decreasing the site binding affinity to zero. By increasing n while decreasing k in inverse linear proportion, the form of the indirect model obtains from the MWC model in the limit $n \to \infty$. For inhibition ($\beta > 0$), set $\beta = nk_t$; then $\lim_{n\to\infty}(1 + k_t x)^n = \lim_{n\to\infty}(1 + \beta x/n)^n = e^{\beta x}$, and Eq. 14a becomes identical to Eq. 6a. Similarly, for activation ($\beta < 0$), Eq. 14b reduces to Eq. 6b by setting $\beta = -nk_r$ and again taking the limit $n \to \infty$.

CONCLUSIONS

For many intrinsic membrane proteins, there is little direct evidence of the existence of binding sites for membrane-soluble molecules known to have a strong influence on protein activity. The form of the titration curve predicted from a general class of indirect mechanisms is similar to the predictions of multisite binding models that exhibit cooperativity based on coupling of ligand binding to a conformational transition. Thus the agreement of experimental dose-response data with predictions of cooperative binding models is not evidence of the existence of binding sites, and inferred binding properties (number of sites, degree of cooperativity, and affinities) may be meaningless.

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