Electrostatic coupling to pH-titrating sites as a source of cooperativity in protein–ligand binding

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Abstract

This paper describes an alternative mechanism for the cooperative binding of charged ligands to proteins. The ligand-binding sites are electrostatically coupled to protein side chains that can undergo protonation and deprotonation. The binding of one ligand alters the protein's protonation equilibrium in a manner that makes the binding of the second ligand more favorable. This mechanism requires no conformational change to produce a cooperative effect, although it is not exclusive of conformational change. We present a theoretical description of the mechanism, and calculations on three kinds of systems: A model system containing one protonation site and two ligand-binding sites; a model system containing two protonation sites and two ligand-binding sites; and calbindin D_{9k}, which contains two Ca²⁺-binding sites and 30 protonation sites. For the one-protonation-site model, it is shown that the influence of the protonation site can only be cooperative. The competition of this effect with the anticooperative effect of ligand–ligand repulsion is studied in detail. For the two-protonation site model, the effect can be either cooperative or, in special cases, anticooperative. For calbindin D_{9k}, the calculations predict that six protonation sites in or near the ligand-binding sites make a cooperative contribution that approximately cancels the anticooperative effect of Ca²⁺–Ca²⁺ repulsion, accounting for more than half of the total cooperative effect that is needed to overcome repulsion and produce the net cooperativity observed experimentally. We argue that cooperative mechanisms of the kind described here are likely when there is more than one ligand-binding site in a protein domain.

Keywords: allostery, calbindin D_{9k} cooperativity, electrostatics, linked equilibrium, multisite titration, pH titration of proteins, pK_a values in proteins

This paper demonstrates that cooperativity in the binding of charged ligands by proteins can arise from electrostatic couplings of the ligand-binding sites to sites of protonation/deprotonation, such as the ionizable side chains of the protein. We provide a theoretical framework for cooperative effects of this kind, and we present calculations on some simple model systems and on the Ca²⁺-binding protein, calbindin D_{9k}. In contrast to traditional models of cooperativity (Monod et al., 1965; Koshland et al., 1966), conformational changes in the protein are not required in our model, and therefore are not included in the present calculations. We study the conditions that cause the effects of electrostatic coupling to protonation sites to be cooperative or anticooperative, and of greater or lesser magnitude. The conditions under which this mechanism can produce a cooperative effect strong enough to overcome the anticooperative effect of direct ligand–ligand repulsion are given particular attention.

We believe that effects of the kind described here are present in many cases of cooperative binding of charged ligands to proteins,

because proteins generally have a large number of ionizable pH-titrating sites spread over their surfaces. Some are even located in the ligand-binding sites where they provide part of the ligation shell. Similar effects could also occur in the binding of neutral ligands if ligand binding is coupled to the release or uptake of protons.

Background

The binding of a ligand *L* to a protein is said to be cooperative if the protein has more than one binding site for *L*, and if the binding of *L* to one or more sites increases the binding affinity of the remaining sites. If the binding affinity of the remaining sites is reduced, the binding is said to be anticooperative, and if the binding of *L* at one site has no effect on the binding affinity of the others, the binding is independent.

All three situations can be encompassed in a description of cooperativity in terms of free energy (Wyman, 1964; Schellman, 1975; Forsén & Linse, 1995). If a protein *P* has two sites at which *L* can bind, the *L*-binding state of *P* can be denoted by a two-digit binary code in which a 0 or a 1 represents unoccupied or occupied ligand site. As an example of this notation, the protein with no

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ligands bound is $P(00)$, the protein with a ligand bound to the first site, but not the second, is $P(01)$, and the standard free energy for binding a ligand to the first site of the unliganded protein is $\Delta G^\circ(00 \rightarrow 01)$. The free energy of cooperativity between the two ligand-binding sites is defined as the difference between the free energy of binding a ligand to the first site with the second site occupied, and the free energy of binding to the first site with the second unoccupied. In our notation, this is

$$\begin{aligned}\Delta\Delta G_c &= \Delta G^\circ(10 \rightarrow 11) - \Delta G^\circ(00 \rightarrow 01) \\ &= \Delta G^\circ(01 \rightarrow 11) - \Delta G^\circ(00 \rightarrow 10)\end{aligned}\quad (1)$$

where the second, equivalent definition follows from binding L to the same sites in a different order. Cooperativity then corresponds to negative values of $\Delta\Delta G_c$, anticooperativity corresponds to positive values of $\Delta\Delta G_c$, and if the sites are independent, $\Delta\Delta G_c$ is zero. This definition of cooperativity in terms of microscopic binding events is the same as that discussed elsewhere (Ackers et al., 1983; Forsén & Linse, 1995). Cooperativities in the range of $-\Delta\Delta G_c = 3$ to 10 kJ/mol are sufficient to give rise to the steep binding isotherms characteristic of cooperative binding (Weber, 1975; Forsén & Linse, 1995). Sometimes it is more convenient to write the above definition in terms of microscopic binding constants rather than the corresponding binding free energies³:

$$\Delta\Delta G_c = -RT \ln(K_{I,II}/K_I) = -RT \ln(K_{II,I}/K_{II}), \quad (2)$$

where K_I and K_{II} are the microscopic binding constants for filling site I or II, respectively, with the other site empty, and $K_{I,II}$ and $K_{II,I}$ are the microscopic binding constants for filling site I or II, with the other site occupied. Equations 1 and 2 are fully equivalent through relations such as $K_I = e^{-\Delta G^\circ(00 \rightarrow 01)/RT}$.

Rather than the microscopic definition above, it is also possible to define cooperativity in terms of macroscopic binding constants, which is very useful in the analysis of experimental data since the macroscopic constants can be obtained from the total ligand-binding isotherms. However, this definition is not equivalent to the microscopic $\Delta\Delta G_c$ defined above. The microscopic and macroscopic formulations of cooperativity have been discussed and compared in depth by several authors (Ackers et al., 1983; Hill, 1985; Di Cera, 1995). Here, we outline the macroscopic formulation, its relation to the microscopic, and our reasons for preferring the microscopic definition. For a macromolecule with two identical and independent sites, one expects that K_1 (the macroscopic binding constant for going from an unligated to a singly ligated state) should be twice the intrinsic binding constant of an individual site, since the ligand can bind either of two sites; and K_2 (the constant for going from the singly to the doubly ligated form) should be half the intrinsic binding constant. Thus the ratio K_2/K_1 should be 1/4, independent of the intrinsic binding constant of the sites. If this ratio is greater than expected the binding is said to be cooperative, and if it is less the binding is anticooperative (Sackett & Saroff, 1996). If a free energy of cooperativity is to be defined, the natural choice is then

$$\text{macroscopic cooperativity} = -RT \ln\left(4 \frac{K_2}{K_1}\right). \quad (3)$$

This differs from the microscopic definition of Equation 2 by

$$-RT \ln \frac{4K_2}{K_1} + RT \ln \frac{K_{II,I}}{K_{II}}.$$

Substituting the relation between the microscopic and macroscopic constants,

$$K_1 = K_I + K_{II} \quad \text{and} \quad K_2 = \frac{K_I K_{II,I}}{K_I + K_{II}},$$

one finds that the difference depends only on the ratio, K_{II}/K_I . The result can be conveniently written

$$\Delta\Delta G_c = -RT \ln\left(4 \frac{K_2}{K_1}\right) - RT \ln\left(\frac{(\eta + 1)^2}{4\eta}\right), \quad (4)$$

where $\eta = K_{II}/K_I$. When both sites are identical, which is the situation upon which the macroscopic definition is based, $\eta = 1$, so that the η -dependent term in Equation 4 is zero and the macroscopic and microscopic definitions become equivalent. However, in the more general case of nonidentical binding sites, the η -dependent term is negative so that $\Delta\Delta G_c < -RT \ln(4K_2/K_1)$; in other words, the macroscopic definition provides an upper limit on the value of the microscopic $\Delta\Delta G_c$ (Linse et al., 1988). It is therefore possible that, in systems with nonidentical sites that bind ligands independently ($\Delta\Delta G_c = 0$), the macroscopic definition would lead to the conclusion that the binding is anticooperative. For the purposes of this paper, we have chosen the microscopic definition because it is more natural for studies focusing on specific molecular mechanisms of cooperativity (Hill, 1985), and because it is more suitable for non-identical sites, such as those in calbindin D_{9k} . Furthermore, Ackers et al. (1983) have shown that it is generally possible to extract the value of $\Delta\Delta G_c$ from individual-site binding isotherms; and estimates of $\Delta\Delta G_c$ for calbindin D_{9k} are becoming available from spectroscopic and mutagenesis studies (Linse et al., 1991). Finally, it will be shown that the main results of this paper would be the same, even if the macroscopic definition were used.

Cooperative effects have usually been explained by mechanisms involving conformational change. For example, Monod et al. (1965) proposed an equilibrium of two protein conformers: T , which has a low ligand-binding affinity at all of its sites, and R , which has a higher affinity at all sites. Ligand binding to any site shifts the R -to- T equilibrium in the direction of R , which increases the affinity for further ligand binding. An alternative model for cooperativity and the Bohr effect in hemoglobin was formulated (Saroff, 1970) that had couplings between the Bohr protonation sites and the oxygen binding sites so that "the cooperative effect [is] generated by the Bohr effect itself." However, the couplings in the model were presumed to arise through a conformational mechanism, rather than electrostatic interactions. More recently, calculations based on a similar model have been presented (Saroff, 1991) showing that cooperativity in either ligand binding or proton binding can arise where clusters of ionizable groups are near ligand-binding sites.

The model of cooperativity to be presented here involves multiple ligand-binding sites, multiple protonation sites, and electro-

³In the present work, the free energy formulation is generally preferred since it provides a more natural framework for physical models of site-site interactions as sums of pairwise-additive potentials.

static interactions between all of these sites. This is closely related to the problem of calculating microscopic protonation state equilibria in proteins that have a large number of protonation sites (Bashford & Karplus, 1991; Beroza et al., 1991; Gilson, 1993). Interest in multisite titration has been stimulated by advances in electrostatic calculations of the intrinsic pH-titration properties of ionizable groups in proteins and their electrostatic interactions with one another (Bashford & Karplus, 1990; Yang et al., 1993). These electrostatic calculations on proteins use the model of Macroscopic Electrostatics with Atomic Detail (MEAD), in which the protein is a low-dielectric object containing embedded point charges and immersed in a high-dielectric medium (water). The charges are atomic partial charges located at the nuclear coordinates and the dielectric boundary is determined by coordinates and atomic radii. The resulting electrostatic equations are solved by finite numerical methods. In this paper we have adapted these methods to the calculation of cooperative effects in calbindin D_{9k} .

Calbindin D_{9k} is a monomeric globular protein of 75 amino acids that cooperatively binds two Ca^{2+} ions per protein molecule (Linse et al., 1987). It is a member of the calmodulin superfamily of proteins containing "EF hand" Ca^{2+} -binding sites, which are conventionally labeled I and II. The two EF hands in calbindin D_{9k} are asymmetric and are both in the same polypeptide chain. Unlike some of the larger members of the calmodulin family, calbindin D_{9k} does not undergo large conformational changes upon ligand binding. Changes are mostly localized to the Ca^{2+} binding sites, although there is some cross-talk between the sites (Carlström & Chazin, 1993; Akke et al., 1995). In measuring the cooperativity of the Ca^{2+} binding of calbindin D_{9k} at low ionic strength, a $-\Delta\Delta G_c$ value of 7.7 ± 1.5 kJ/mol was found (Linse et al., 1991). In the X-ray crystallographic structure (Svensson et al., 1992), the Ca^{2+} -binding sites are only 11.4 Å apart, so there should be a substantial electrostatic repulsion between the two Ca^{2+} ions in the fully loaded state. An explanation of cooperativity must account for the free energy to overcome both the anticooperative effect of this repulsion and the 7.7 kJ/mol of the observed net cooperativity. There are several carboxylic acid groups within, and between, the binding sites, and we will show that much of the total cooperative influence can be accounted for by electrostatic couplings involving these groups.

Sketch of the model and applications

The mechanism for cooperativity to be described in this paper is diagrammed in Figure 1. There are two ligand-binding sites, and N sites whose protonation states are in equilibrium with the pH of the solution. Four different ligand-binding states, and 2^N different protonation states, are possible. The four ligand-binding states shown in the diagram all have the same protein conformation, and if the sites of protonation were absent, the electrostatic repulsion between the like-charged ligands (large filled circles) would result in anticooperativity (positive $\Delta\Delta G_c$). However, the ensemble of protonation states, represented by the set of small grey circles in Figure 1, is different in each ligand state due to the electrostatic couplings between the ligand sites and the protonation sites. These couplings in turn affect the affinities of the ligand-binding sites. The protonation sites are also electrostatically coupled to each other.

In the next section, we present a theoretical model of this mechanism of cooperativity. The Results section describes the application of the model to three different systems. The first is the simplest

Fig. 1. A protein with two sites for binding the charged ligand L and N protonation sites. The states of the ligand-binding sites are indicated by a binary code and by the large open (unbound) or closed (bound) circles. For each of the four possible ligand states, there is an ensemble of states for the proton-binding sites (grey circles), which lead to pH dependence of the ΔG° .

possible model, a system with one protonation site and two ligand-binding sites, and it is analyzed in some detail. The second model is the next simplest possible model: two protonation sites and two ligand-binding sites. The third is calbindin D_{9k} . In the Discussion, we summarize the important points of these results and present some general comments about the cooperative mechanism. Finally, a Methods section contains the technical details of electrostatic calculations and protein models.

Theory of cooperative ligand binding and multisite protonation

Binding of ligand L only

We begin with a treatment of ligand binding without any consideration of proton binding, but we develop a formalism similar to that used for multisite pH titration problems. This will allow pH dependence to be introduced easily in later subsections.

In the limit of dilute macromolecules, the activity of each macromolecular microstate can be identified with its population, and one has relationships such as

$$[P(01)]/[P(00)] = e^{-\beta\Delta G^\circ(00 \rightarrow 01) + \ln a_L} \quad (5)$$

where a_L is the activity of the ligand L and $\beta = (RT)^{-1}$. We adopt the convention that G° is defined in relation to the reference state $P(00)$ so that, for example, $G^\circ(01) \equiv \Delta G^\circ(00 \rightarrow 01)$. Denoting ligation microstates by a two-dimensional vector $\mathbf{c}$, whose elements can take the values 0 or 1, the fraction F of protein in a particular microstate $\mathbf{c}$ can be obtained by a generalization of relations such as Equation 5:

$$F(\mathbf{c}) = \frac{e^{-\beta G^\circ(\mathbf{c}) + N_L(\mathbf{c}) \ln a_L}}{Z_L(a_L)}, \quad (6)$$

where N_L is the number of bound ligands in microstate $\mathbf{c}$ and Z_L is a normalization constant. The G° for the two-site case can be expressed in a functional form involving only three parameters: Two free energies of binding ligand to one site while the other is unligated, and the energetic coupling between binding events at the two sites, W_{L_1, L_2} .

$$G^\circ(\mathbf{c}) = c_1 \Delta G_{a,1} + c_2 \Delta G_{a,2} + c_1 c_2 W_{L_1, L_2}. \quad (7)$$

The ΔG_a are called the intrinsic binding free energies of sites 1 and 2 (Ackers et al., 1983). The last term corresponds to the cooperativity, that is, $\Delta \Delta G_c = W_{L_1, L_2}$. There is no loss of generality in this formulation since the two-site case has only four binding microstates and thus, no more than three physically significant independent energy differences, and Equation 7 has three independent parameters. Although cooperativity formally appears as an interaction between the ligands, a much wider variety cooperative or anticooperative mechanisms can be accommodated in the same formalism (Hill, 1985).⁴

If there is no conformational change, and the interaction between the sites is only the electrostatic interaction between identically charged ligands, W_{L_1, L_2} is positive and the effect is anticooperative. The cooperativity or anticooperativity is independent of the intrinsic binding free energies ΔG_a . This is an advantage of the definition of cooperativity given in Equation 1 (Forsén & Linse, 1995); it will continue to hold, even when coupling to protonation sites is introduced.

Protonation/deprotonation only

Consider the problem of a protein having N ionizable groups that can undergo protonation or deprotonation in equilibrium with the solution pH. No binding of other ligands is considered. There are 2^N possible protonation microstates, and they can be denoted by an N -component vector $\mathbf{x}$, whose elements take on the values 0 or 1 according to whether the corresponding proton binding site is deprotonated or protonated.

A functional form can be chosen for $G^\circ(\mathbf{x})$ that is analogous to Equation 7 for $G^\circ(\mathbf{c})$, in that it consists of one-site terms representing the intrinsic binding affinities of the sites, and two-site terms representing site–site interactions. This choice is not completely general for the N -site case in that it contains no three-site or higher terms; but it is consistent with the picture of a protein in a single conformation whose sites interact electrostatically. To describe the sites' intrinsic binding affinities in terms similar to the pK_a values of model compounds, it is conventional to use the "intrinsic pK_a " or pK_{int} , which is defined as the pK_a that the site would have if all other sites were held in their electrically neutral protonation states (Tanford & Kirkwood, 1957). One then has

⁴Some authors, including Hill (1985), occasionally use notations with special factors or terms to single out interactions that differentiate otherwise identical sites, or the influence of an empty site with a binding process at another site. This is mostly a matter of notational convenience, and we prefer to let differentiating or empty-site interactions be carried in the intrinsic binding free energies, as does Di Cera (1995). Our definition of the intrinsic binding energy should not be confused with some of the definitions in notations with interaction factors, where the intrinsic binding constants are sometimes defined as the noninteraction factors of certain microscopic binding constants (Sackett & Saroff, 1996).

$$G^\circ(\mathbf{x}) = -2.3\beta^{-1} \sum_{\mu}^N x_{\mu} pK_{\text{int},\mu} + \frac{1}{2} \sum_{\mu}^N \sum_{\nu}^N (x_{\mu} + q_{\mu}^{\circ})(x_{\nu} + q_{\nu}^{\circ}) W_{\mu\nu}, \quad (8)$$

where the Greek indices label the sites, $W_{\mu\nu}$ is the interaction between two unit charges placed at sites μ and ν , and the q° are the charges of the sites in their deprotonated states. (For convenience, we have written 2.3 for the value of $\ln 10$, but in actual calculations, a more precise value is used.) By adding a suitable constant, the expression for $G^\circ(\mathbf{x})$ could be made to go to zero for a particular protonation reference state, such as the fully deprotonated state or the state with all sites neutral, but we have omitted any such constant since it simplifies writing and does not affect any physically meaningful quantity.

The expression for the fraction of the protein in a particular microstate is analogous to Equation 6:

$$F(\mathbf{x}) = \frac{e^{-\beta G^\circ(\mathbf{x}) - 2.3 N_H(\mathbf{x}) \text{pH}}}{Z_H(\text{pH})} \quad (9)$$

where the normalization constant, Z_H involves a sum over all 2^N possible protonation microstates (Bashford & Karplus, 1991). The fractional protonation of a particular site i is obtained by summing over all states in which i is protonated,

$$\theta_i = \sum_{\mathbf{x}}^N x_i F(\mathbf{x}) \quad (10)$$

where the summation notation indicates a sum over all 2^N microstates.

Coupling ligand binding and protonation

We now consider a protein that includes both ligand-binding sites and protonation sites and with pairwise couplings between all sites. An expression for $G^\circ(\mathbf{c}, \text{pH})$, the pH-dependent standard free energy of a protein in a specified ligand microstate and an equilibrium distribution of protonation states, is needed for the description of pH-dependent cooperativity.

The starting point is a microscopic free energy expression in which the protein microstate is specified by both the ligand-binding vector $\mathbf{c}$ and the proton-binding vector $\mathbf{x}$. This expression is

$$G^\circ(\mathbf{c}, \mathbf{x}) = c_1 \Delta G_{a,1} + c_2 \Delta G_{a,2} + c_1 c_2 W_{L_1, L_2} - 2.3\beta^{-1} \sum_{\mu}^N x_{\mu} pK_{\text{int},\mu} + \frac{1}{2} \sum_{\mu}^N \sum_{\nu}^N (x_{\mu} + q_{\mu}^{\circ})(x_{\nu} + q_{\nu}^{\circ}) W_{\mu\nu} + \sum_{\mu}^N c_1 x_{\mu} W_{L_1\mu} + \sum_{\mu}^N c_2 x_{\mu} W_{L_2\mu}, \quad (11)$$

which is a combination of Equations 7 and 8, with the last two terms added to introduce the coupling between proton-binding and ligand-binding sites. An expression similar to Equation 6 or 9 for the fractional population, $F(\mathbf{c}, \mathbf{x})$ of a given ligand–protonation microstate could be written, but, as suggested by Figure 1, the

quantity of interest here is the pH-dependent total population of a given ligand state over an equilibrium ensemble of protonation states. This is obtained by summing the expression for $F(\mathbf{c}, \mathbf{x})$ over all possible protonation states:

$$F(\mathbf{c}, \text{pH}) = \frac{\sum_{\mathbf{x}} e^{-\beta G^{\circ}(\mathbf{c}, \mathbf{x}) + N_L(\mathbf{c}) \ln a_L - 2.3 N_H(\mathbf{x}) \text{pH}}}{Z_{LH}(a_L, \text{pH})}. \quad (12)$$

As one can verify by substitution,

$$F(\mathbf{c}, \text{pH}) = \frac{e^{-\beta G^{\circ}(\mathbf{c}, \text{pH}) + N_L(\mathbf{c}) \ln a_L}}{Z'_L(a_L, \text{pH})}, \quad (13)$$

where the pH-dependent free energy for a ligand state is

$$G^{\circ}(\mathbf{c}, \text{pH}) = -\beta^{-1} \ln \left[\sum_{\mathbf{x}} e^{-\beta G^{\circ}(\mathbf{c}, \mathbf{x}) - 2.3 N_H(\mathbf{x}) \text{pH}} \right]. \quad (14)$$

The identification of Equation 14 as a free energy is supported both by its relation to equilibrium constants through Equation 13, and by its functional form, which is that of a canonical ensemble expression in which the “Hamiltonian” is $G^{\circ}(\mathbf{c}, \mathbf{x}) + 2.3\beta^{-1}N_H(\mathbf{x})\text{pH}$, which is the free energy to bring a protein in ligand state $\mathbf{c}$ from a reference protonation state to protonation state $\mathbf{x}$ at a specified pH. Similar expressions have appeared in studies of approximation methods for the multisite titration problem (Bashford & Karplus, 1991). Substituting Equation 11 into Equation 14, factoring out the pH-independent parts, and using the fact that $N_H(\mathbf{x}) = \sum_{\mu} x_{\mu}$ yields a formula expressing how the linkage to protons modifies the value of $G^{\circ}(\mathbf{c})$ relative to the no-proton formula, Equation 7:

$$\begin{aligned} G^{\circ}(\mathbf{c}, \text{pH}) &= c_1 \Delta G_{a,1} + c_2 \Delta G_{a,2} + c_1 c_2 W_{L_1, L_2} \\ &- \beta^{-1} \ln \left[\sum_{\mathbf{x}} \exp \left(2.3 \sum_{\mu} x_{\mu} (\text{p}K_{\text{int}, \mu} - \text{pH}) \right. \right. \\ &\quad \left. \left. - \beta \frac{1}{2} \sum_{\mu} \sum_{\nu} (x_{\mu} + q_{\mu}^{\circ})(x_{\nu} + q_{\nu}^{\circ}) W_{\mu\nu} \right. \right. \\ &\quad \left. \left. - \beta \sum_{\mu} x_{\mu} (c_1 W_{L_1\mu} + c_2 W_{L_2\mu}) \right) \right]. \quad (15) \end{aligned}$$

pH-dependent cooperativity

Using the above results, an expression for cooperativity as a function of pH can be given in which interactions between proton-binding and ligand-binding sites are accounted for:

$$\begin{aligned} \Delta\Delta G_c(\text{pH}) &= G^{\circ}(11, \text{pH}) - G^{\circ}(10, \text{pH}) \\ &- G^{\circ}(01, \text{pH}) + G^{\circ}(00, \text{pH}). \quad (16) \end{aligned}$$

Before writing out a more explicit expression for the cooperativity, it is convenient to introduce dimensionless quantities:

$$p_{\mu} \equiv 2.3(\text{p}K_{\text{int}, \mu} - \text{pH}) - \beta \sum_{\nu} q_{\nu}^{\circ} W_{\mu\nu}$$

$$g_{\mu\nu} \equiv \beta W_{\mu\nu}$$

$$z \equiv \text{valence of ligand ion}$$

$$zw_{L_1, \mu} \equiv \beta W_{L_1\mu}$$

$$zw_{L_2, \mu} \equiv \beta W_{L_2\mu}$$

$$z^2 w_{L_1, L_2} \equiv \beta W_{L_1, L_2}. \quad (17)$$

The w and g values are always positive, z is a nonzero integer, and the pH dependence is carried in the p_{μ} . We also adopt the physicists' convention that repeated indices within a term imply summation over those indices (e.g., $p_{\mu} x_{\mu} \equiv \sum_{\mu} p_{\mu} x_{\mu}$). The expression for cooperativity then becomes

$$\begin{aligned} \beta\Delta\Delta G_c(\text{pH}) &= z^2 w_{L_1, L_2} \\ &- \ln \sum_{\mathbf{x}} e^{x_{\mu} p_{\mu} - z x_{\mu} (w_{L_1, \mu} + w_{L_2, \mu}) - \frac{1}{2} x_{\mu} x_{\nu} g_{\mu\nu}} \\ &+ \ln \sum_{\mathbf{x}} e^{x_{\mu} p_{\mu} - z x_{\mu} w_{L_2, \mu} - \frac{1}{2} x_{\mu} x_{\nu} g_{\mu\nu}} \\ &+ \ln \sum_{\mathbf{x}} e^{x_{\mu} p_{\mu} - z x_{\mu} w_{L_1, \mu} - \frac{1}{2} x_{\mu} x_{\nu} g_{\mu\nu}} \\ &- \ln \sum_{\mathbf{x}} e^{x_{\mu} p_{\mu} - \frac{1}{2} x_{\mu} x_{\nu} g_{\mu\nu}}. \quad (18) \end{aligned}$$

As in the ligands-only case, the cooperativity expression, Equation 18, is independent of the intrinsic binding affinities of the ligand-binding sites.

In many proteins, the number of titrating sites N is well above 20 which makes it impractical to carry out the summation over 2^N protonation vectors indicated in Equation 18. Several approximation schemes have been worked out that allow one to calculate the total protonation, or the protonation θ_i , of particular sites as a function of pH in such situations (Bashford & Karplus, 1991; Beroza et al., 1991; Gilson, 1993; Yang et al., 1993). It is then possible to use an alternative formalism for the pH dependence of $G^{\circ}(\mathbf{c}, \text{pH})$ based on integration over a binding isotherm:

$$G^{\circ}(\mathbf{c}, \text{pH}) - G^{\circ}(\mathbf{c}, \text{pH}_0) = 2.3\beta^{-1} \int_{\text{pH}_0}^{\text{pH}} \sum_i \theta_i(\text{pH}') d\text{pH}'. \quad (19)$$

The consistency of this formulation with the foregoing can be verified by differentiating Equation 14 with respect to pH, and comparing the result to Equation 10. The cooperativity is then

$$\beta\Delta\Delta G_c(\text{pH}) = z^2 w_{L_1, L_2} + 2.3 \sum_i \int_{\text{pH}_0}^{\text{pH}} \Delta\Delta\theta(\text{pH}) d\text{pH}$$

$$\begin{aligned} \Delta\Delta\theta(\text{pH}) &\equiv \theta_i(11, \text{pH}) - \theta_i(10, \text{pH}) \\ &- \theta_i(01, \text{pH}) + \theta_i(00, \text{pH}), \end{aligned} \quad (20)$$

where it is assumed that pH_0 is taken at an extreme value such that the protein is either fully protonated or fully deprotonated so that $\Delta\Delta G_c(\text{pH}_0) = W_{L_1, L_2}$. A useful feature of this formula is that the terms in the sum can be taken as an indication of the importance of the proton binding site i to the cooperativity of the binding of the ligand L . Strictly speaking, these terms cannot be taken as “the contribution of site i to cooperativity,” since it is only the total of the θ_i , and not the individual terms, which are exact differentials. Indeed, the choice of either very high or very low pH_0 will give different results for the individual terms, but the same result for their sum. The situation is analogous to that of the thermodynamic integration method in molecular mechanics (McQuarrie, 1976) in which the total work against the mean force is independent of the path of integration, but the contribution of individual force terms to the integral is not.

Results

One protonation site and two ligand-binding sites

The case of one protonation site and two ligand-binding sites is simple enough to be studied analytically. We can show that the effect of the protonation site is always cooperative, and we can explore the limiting case of the protonation site very near one of the ligand-binding sites without any extra assumptions. Then by introducing some simple assumptions about the relationship of site–site interactions to geometry, we can produce a series of plots showing how cooperativity is related to geometry, ligand charge, and pH.

Since there is only one protonation site, the g matrix terms in Equation 18 do not occur, the writing of the index μ can be dropped, and the sum over the two possible protonation states can be written explicitly. The expression for cooperativity then simplifies to

$$\begin{aligned} \beta\Delta\Delta G_c(\text{pH}) &= z^2 w_{L_1, L_2} - \ln(1 + e^{p-z(w_{L_1}+w_{L_2})}) \\ &+ \ln(1 + e^{p-zw_{L_2}}) + \ln(1 + e^{p-zw_{L_1}}) \\ &- \ln(1 + e^p). \end{aligned} \quad (21)$$

The first term is the (anticooperative) ligand–ligand repulsion, and the logarithmic terms contain the protonation site’s contribution to cooperativity. Since the pH dependence enters only through the single variable $p = 2.3(\text{pK}_{\text{int}} - \text{pH})$, plots relevant for any choice of pK_{int} can be made by taking $\text{pH} - \text{pK}_{\text{int}}$ as the independent variable. Neither p , nor Equation 21, contains any dependence on q° , so the same plots will apply to both cationic residues such as lysine or anionic residues such as glutamate. The remaining parameters that can lead to different forms of the plots are the three coupling parameters, w_{L_1, L_2} , w_{L_1} , and w_{L_2} , and the ligand valence, z .

One can prove that the protonation site can only have a cooperative influence by combining and rearranging the logarithmic terms in Equation 21:

$$- \ln \left[1 + \frac{e^p (1 - e^{-zw_{L_1}})(1 - e^{-zw_{L_2}})}{1 + e^{p-zw_{L_1}} + e^{p-zw_{L_2}} + e^{2p-z(w_{L_1}+w_{L_2})}} \right].$$

Since w_{L_1} and w_{L_2} are positive by definition, the large fraction in the logarithm will always be positive, so that the contribution of the logarithmic term to $\Delta\Delta G_c$ is always negative. Differentiating Equation 21 with respect to p , and setting the partial derivative to zero, gives the result that there is a single critical point in the p dependence of $\Delta\Delta G_c$, which is located at $p = z(w_{L_1} + w_{L_2})/2$. It can be shown that this is always a minimum. As for asymptotic behavior, the logarithmic part goes to zero for large negative or positive values of p . Thus, we find that the effect of a single proton-binding site interacting electrostatically with two ionic ligand-binding sites is to increase the cooperativity of ligand binding. This effect is strongest at a pH of

$$\text{pH}_{\text{min}} = \text{pK}_{\text{int}} - z(w_{L_1} + w_{L_2})/4.605, \quad (22)$$

and vanishes at extremes of pH. This result holds regardless of ion valence or the valence of the charged form of the pH-titrating group.

An intuitive explanation of this result can be made that is similar to the traditional R–T equilibrium model of cooperativity. The binding of the first ligand changes the protonation equilibrium in a manner that lowers its energy of interaction with the protonation site. Since the second ligand has the same charge, its energy of interaction with the protonation site will also be lowered by this change of protonation, so that the binding of the second ligand becomes more favorable. The argument holds regardless of whether the interaction between the ligand and the charged form of the protonation site is attractive or repulsive. This result is consistent with earlier findings (Weber, 1975), in which the case of two noninteracting Y-binding sites having equal interactions with an X-binding site was analyzed.

The manner in which the cooperative effect of the protonation site depends on its placement in the protein can be explored through the w dependence of Equation 21. The partial derivative of $\Delta\Delta G_c$ with respect to either w_{L_1} or w_{L_2} is negative for either sign of z . Assuming that the w increase as the corresponding site–site distances decrease, this means that a repositioning of the protonation site so that it is closer to both ligand sites increases the cooperativity, as one might have expected. At some point, it becomes impossible to move the protonation site so as to increase the interaction with one ligand site without reducing its interaction with the other. Therefore, we shall consider the effect of moving “between” the two sites.

First consider the extreme in which the protonation site is very close to ligand site 1. In this case, w_{L_1} becomes very large, and w_{L_2} becomes identical to w_{L_1, L_2} (since a unit charge at the protonation site has the same effect on other sites as if it were placed on ligand site 1). If the charge z of the ligand L is positive, terms in Equation 21 involving $e^{-zw_{L_1}}$ will vanish. Substituting w_{L_1, L_2} for w_{L_2} , and considering the limits of high and low pH (low and high p) one finds

$$\begin{aligned} \beta\Delta\Delta G_c &= z^2 w_{L_1, L_2} - \ln \left[\frac{1 + e^p}{1 + e^{p-zw_{L_1, L_2}}} \right] \\ &\rightarrow \begin{cases} (z^2 - z)w_{L_1, L_2}, & \text{low pH} \\ z^2 w_{L_1, L_2}, & \text{high pH.} \end{cases} \end{aligned} \quad (23)$$

The behavior of $\Delta\Delta G_c$ between the two pH limits is monotonic. The low pH “limit” here must be understood with the proviso that the value of p not be so extreme as to invalidate the neglect of the $e^{-zw_{L_1}}$ terms; in other words, one must have $p \ll w_{L_1}$. As p is increased beyond w_{L_1} , the value of $\Delta\Delta G_c$ approaches its ultimate low-pH limit of $z^2 w_{L_1, L_2}$ and the “limit” referred to in Equation 23 becomes part of a broad, flat minimum.

An intuitive understanding of Equation 23 can be gained by considering the $z = +1$ case. At low pH a protein with ligand site 1 empty will have the protonation site occupied. The binding of a ligand to site 1 will displace the nearby proton because of the very strong interaction, w_{L_1} (assuming that $p \ll w_{L_1}$), so that ligand site 2 “sees” no net change of charge from the region of site 1 and $\Delta\Delta G_c$ is zero (independent binding). If z is $+2$ or more, the protonation site’s effect can only cancel one unit of charge, so the other ligand site sees $z - 1$ of the first ligand’s charge, and the effect of the protonation site is to reduce, but not eliminate, the anticooperativity.

If the ligand charge z is negative, similar considerations to those above lead to the result that $\beta\Delta\Delta G_c$ tends to $z^2 w_{L_1, L_2}$ in the low pH limit, and decreases monotonically with increasing pH to the limiting value, $z^2 w_{L_1, L_2} - z w_{L_1, L_2}$.

To further explore the situation in which the protonation site has various possible positions that are “between” the ligand-binding sites (in the sense that increasing the interaction with one reduces the interaction with the other), we adopt the geometric model shown in Figure 2. The protonation site can be located anywhere along a path between the ligand sites. Motion along the path is parameterized by the variable λ whose value ranges from -1 to 1 as the protonation site is moved from ligand site 1 to ligand site 2. The couplings w_{L_1} and w_{L_2} are then functions of λ . In accord with the notion of “between” and the end-point cases analyzed above, $w_{L_1}(\lambda)$ approaches $+\infty$ as λ goes to -1 and decreases monotonically to w_{L_1, L_2} as λ goes to $+1$. Similarly, $w_{L_2}(\lambda)$ is w_{L_1, L_2} at $\lambda = -1$ and monotonically increases toward $+\infty$ as λ goes to $+1$.

One can begin to explore the extremes of cooperative behavior along the path by further assuming that the symmetry of the end point conditions extends along the whole path, that is $w_{L_1}(\lambda) = w_{L_2}(-\lambda)$. Using this symmetry relation,

$$\frac{d\Delta\Delta G_c}{d\lambda} = \frac{dw_{L_1}}{d\lambda} \left[\frac{\partial\Delta\Delta G_c}{\partial w_{L_1}} - \frac{\partial\Delta\Delta G_c}{\partial w_{L_2}} \right]. \quad (24)$$

Because of the symmetry of $\Delta\Delta G_c$ with respect to the interchange of w_{L_1} and w_{L_2} , this expression goes to zero when the protonation site is exactly in the “middle” (i.e., at $\lambda = 0$ so that $w_{L_1} = w_{L_2}$). Substitution of the explicit form of $\Delta\Delta G_c$ and examination of the second derivatives show that this critical point can be either a

Fig. 2. Model system with two ligand-binding sites and one protonation site. The distance between ligand-binding sites is 10 Å.

minimum, a maximum or an inflection point of $\Delta\Delta G_c$ with respect to λ , depending on the value of p (i.e., the pH) and the functional form of $w_{L_1}(\lambda)$. Combining this with the extremum condition on pH, $p = z(w_{L_1} + w_{L_2})/2$, and writing w for the equal values of w_{L_1} and w_{L_2} gives

$$\beta\Delta\Delta G_c = z^2 w_{L_1, L_2} - \ln \left[\frac{(2 + e^{-zw} + e^{zw})}{4} \right]. \quad (25)$$

If w is sufficiently large, the e^{zw} term will dominate in the logarithm giving the result $\beta\Delta\Delta G_c = z^2 w_{L_1, L_2} - zw + 1.386$. Whether this leads to positive or negative net cooperativity depends on how much larger w is than w_{L_1, L_2} . One expects w to be larger, since the protonation site in the middle position is closer to the ligands than the ligands are to each other. The more rapidly interactions fall off with distance, the more likely is net cooperativity.

To go further, it is necessary to specify a functional form for the dependence of the coupling parameters w_{L_1} and w_{L_2} on the positional parameter λ . We have studied two inverse power laws: coulombic and inverse-square. The interest of the former for electrostatic interactions is clear. The latter is interesting because it is sometimes used to approximate the manner in which the solvent screening of charge–charge interactions in proteins increases with charge separation. In molecular mechanics calculations, the use of distance-dependent dielectric constants proportional to the charge separation amounts to an inverse-square law (Brooks et al., 1983). An inverse-square law has been found to give a good account of the evolutionary optimization of electrostatic interactions in proteins (Spassov et al., 1994). It also gives a reasonable fit to finite-difference solutions to the Poisson equation in the MEAD model (V. Spassov & D. Bashford, unpubl. data). The specific functional forms that meet the symmetry and endpoint requirements detailed above are

$$w_{L_1} = \frac{2w_{L_1, L_2}}{|\lambda + 1|}, \quad w_{L_2} = \frac{2w_{L_1, L_2}}{|\lambda - 1|}, \quad (26)$$

for the Coulombic form, and

$$w_{L_1} = \frac{4w_{L_1, L_2}}{(\lambda + 1)^2}, \quad w_{L_2} = \frac{4w_{L_1, L_2}}{(\lambda - 1)^2}, \quad (27)$$

for the inverse square law. If the ligand sites are 10 Å apart, and a Coulomb law applies with an effective dielectric constant of 40, the value of w_{L_1, L_2} is 1.38. An inverse square law arising from a distance-dependent dielectric, $\epsilon = 4r$, leads to the same w_{L_1, L_2} value. This value corresponds roughly to the ligand–ligand repulsion term calculated from a MEAD model for calbindin D_{9k} (see below).

Plots of $\Delta\Delta G_c$ versus pH with a coulombic law are shown in Figures 3A and 3B. When $z = +1$, the effect of the protonation site is just enough to cancel the anticooperativity of the ligand–ligand interaction. Varying λ changes the pH range, but not the magnitude of the effect. When $z = +2$, the ligand–ligand repulsion is four times greater, and the protonation site’s effect only cancels part of it. As in the $z = +1$ case, variation of λ affects the pH range more than the magnitude of the protonation site’s influence.

When an inverse square law is used, the cooperative influence of the protonation site is much stronger, as one can see in Figures 3C and 3D. The magnitude of the cooperative effect of the protonation

Fig. 3. Plots of $\beta\Delta\Delta G_c$ versus pH at various values of λ . Plot for (A) a Coulomb law, Equation 26, and $z = 1$; (B) a Coulomb law and $z = 2$; (C) an inverse-square law, Equation 27, and $z = 1$; (D) an inverse-square law and $z = 2$.

site can be sufficient not only to cancel the ligand–ligand repulsion, but also to produce net cooperativity. The effect is maximal with the protonation site halfway between the ligand sites ($\lambda = 0$). Moving toward the ends decreases the cooperativity but broadens its pH range. Net cooperativity is more easily achieved with $z = +1$ than with $z = +2$, but is possible in either case.

Two protonation sites and two ligand-binding sites

The case of two protonation sites and two sites binding the ligand L is not as easy to study analytically as the preceding one-protonation site case. The parameters influencing the cooperativity are the pK_{int} and q° values of the two sites, the ligand valence z , the ligand–ligand coupling w_{L_1, L_2} , the four coupling parameters between the ligand sites and the protonation sites, and the coupling between the two protonation sites. The elimination of the pK_{int} and q° dependencies, used in the case of one protonation site, cannot be used here, except under special assumptions.

Even so, a limited analytical study can be made. The most important result is that the effect of the protonation sites is not necessarily cooperative. Starting from Equation 14, and making some lengthy, but straightforward, algebraic manipulations (not shown), we find a much more limited guarantee: The effect will be cooperative if one protonation site’s coupling to both ligand sites is stronger than or equal to that of the other protonation site. In other words, if

$$w_{1\mu} \geq w_{1\nu} \quad \text{and} \quad w_{2\mu} \geq w_{2\nu}, \quad (28)$$

then the effect of the protonation sites will be cooperative regardless of the value of other parameters. If this condition is not met, the effect can be either cooperative or anticooperative, but larger values of the coupling between the two protonation sites tend to favor anticooperativity. Both cooperative and anticooperative cases will be presented below.

A full exploration of the space of eight continuous and three discrete parameters on which cooperativity depends would be too lengthy. Therefore, we will present a smaller set of cases likely to mimic situations found in proteins, particularly Ca^{2+} -binding proteins.

Two types of geometries that can be varied according to a parameter λ are shown in Figure 4. In Figure 4A, both protonation sites are near the center at $\lambda = 0$, and spread apart to be near the ligand sites at $\lambda = 1$. In Figure 4B, the two protonation sites stay close together and move as a pair from the center to one of the ligand-binding sites as λ goes from 0 to 1. As discussed in the previous section, the inverse square law is probably more representative of interactions of charges near the protein surface than a coulomb law, so an inverse square law is used to obtain coupling constants from the geometries of Figure 4. In keeping with the interest in Ca^{2+} binding proteins, the ligands in all of the calculations of this subsection are di-cations ($z = 2$).

Results from calculations based on the geometry of Figure 4A are presented in Figure 5. In the calculations for Figure 5A, the two protonation sites were assigned the same pK_{int} values and both had $q^\circ = 0$ (cationic sites). Taking $\text{pH} - pK_{int}$ as the dependent variable gives a plot independent of the particular value of pK_{int} , as in the

Fig. 4. Model systems with two ligand-binding sites and two protonation sites. The distance between ligand-binding sites is 10 Å.

one-site case. Net cooperativity is seen and the cooperative effects are of about the same magnitude as in the most closely analogous one-site case, that of Figure 3D. However, the cooperativity persists over a wider range of pH values. When each of the protonation sites is near one of the ligand-binding sites ($\lambda = 1$), the situation is similar to that discussed in connection with Equation 23 for the single protonation site placed near a ligand-binding site. At low pH, one proton is displaced upon ligand binding at each of the ligand-binding sites. The effective charge contributing to anticooperativity is reduced from z to $z - 1$. This reduces anticooperative effect of ligand–ligand repulsion by a factor of $(z - 1)^2/z^2$.

The calculations for Figure 5B were the same, except that both protonation sites were assigned $q^\circ = -1$ (anionic sites). As one can see from the definition of p_μ in Equation 17 and the form of Equation 18, changing the value of q° simply shifts the pH curve of cooperativity along the pH axis, but the extent of the shift depends on the coupling between protonation sites. Since this coupling varies with λ in the geometry of Figure 4, each curve in Figure 5B is shifted by a different amount relative to the corresponding curve in Figure 5A.

A calculation intended to correspond to an acid–base pair such as Lys–Asp is presented in Figure 5C. The pK_{int} of one protonation site was set to 4.0, with $q^\circ = -1$ and the other was assigned $pK_{\text{int}} = 10.0$ and $q^\circ = 0$. Since the two pK_{int} values are not the same, pH is used directly as the independent variable. The resulting pH dependence of the cooperativity is more complex than in previous cases. When the protonation sites are both in the center ($\lambda = 0$), a plot with two minima results. Each of these minima resembles the one-protonation-site minimum of Figure 3D, and can be explained simply as one-site cases corresponding to each of the two protonation sites acting alone. At low pH the cationic site with a high pK_{int} value can be regarded as a fixed positive charge. Only the anionic site is “active” in influencing cooperativity. Using the expression, Equation 22, for the pH minimum in the one-site case, and the inverse square law formula with the $\lambda = 0$ geometry, we

Fig. 5. Cooperativity as a function of pH resulting from the geometries of Figure 4A: (A) two cationic groups; (B) two anionic groups; (C) an oppositely charged pair, such as Asp-Lys.

find that the minimum should lie 4.1 pH units below the pK_{int} value of active protonation site. However, the appropriate pK_{int} here is not the original value of 4.0. The influence of the effectively fixed positive charge of the other protonation site must be included. This interaction shifts the effective pK_{int} down by 3.7 units, according to the inverse square law. The minimum is then expected at $4.0 - 4.1 - 3.7 = -3.8$, which is what is seen in Figure 5C. For the high pH part of the curve, the anionic site can be regarded as a fixed negative charge, and the analysis is similar to that above, except that the 3.7 pH-unit influence of the other protonation site now has the opposite sign. The resulting minimum comes out near the original pK_{int} value of 10.

Results from calculations, based on the moving pair geometry of Figure 4B, are presented in Figure 6. The calculations for Fig-

Fig. 6. Cooperativity as a function of pH resulting from the geometries of Figure 4B: (A) two groups of the same charge; (B) a salt bridge of a Lys-like and an Asp-like site. The plots extend beyond the experimentally accessible pH range to show the general trends of the process.

ure 6A are analogous to those from Figure 5A, in that both protonation sites were assigned the same pK_{intr} value and both have the same q^0 . However, since the coupling between protonation sites is the same for all values of λ , changing both q^0 values at once simply shifts all curves along the pH axis by the same amount. Therefore, a plot independent of the choice of both q^0 and pK_{intr} can be produced by using $\text{pH} - pK_{\text{intr}} + q^0 g/2.3$ as the independent variable. The $\lambda = 0$ curve is identical to its counterpart in Figure 5A, but it does not contain a global minimum in $\Delta\Delta G_c$. Stronger cooperative effects are obtained when the pair is slightly shifted from the center. When the pair is shifted to be near one ligand site ($\lambda = 1$), both protons are displaced upon ligand binding at that site at low pH, so that contribution of the ligand charge at that site to anticooperative repulsion is reduced by two units. In the $z = +2$ case, this abolishes the anticooperative effect of ligand–ligand repulsion.

In the calculations for Figure 6B, which represents a salt-bridged pair such as Lys–Asp moving between the two ligand sites, the same pK_{intr} and q^0 values were used as those for Figure 5C. The results are similar to those in Figure 5C, but at the $\lambda = 1$ geometry, there is a full cancellation of anticooperativity rather than a partial cancellation.

In the results presented so far, the effect of the two protonation sites has always been cooperative, even though the analytical studies leading to Equation 28 showed the possibility of anticooperative effects. Based on both analytical and numerical studies, we believe that anticooperative contributions from the protonation sites

Fig. 7. An anticooperative geometry.

should be rare. However, it is possible to construct arrangements involving a pair of protonation sites that are plausible from the point of view of protein structure, and which display anticooperative effects arising from the protonation sites. The geometry for such an arrangement is shown in Figure 7. Both protonation sites have the same pK_{intr} values and both are anionic. The ligand charge is $+2$. The resulting cooperativity plot (Fig. 8) shows that the protonation sites have an anticooperative effect in a pH range below the pK_{intr} of the protonation sites.

Examination of the titration curves of the protonation sites in each of the ligand states (curves not shown) leads to the following explanation of the anticooperative effect: At a pH somewhat below the pK_{intr} values, a protein in the apo ligand state (00) will have both protonation sites protonated, and therefore neutral. When one positively charged ligand binds to form the (01) ligand state, the proton nearest that ligand site is displaced and the ionizable group's negative charge interacts favorably with the ligand. When the second ligand binds to form the (11) state, it is more difficult to obtain a second negative charge for it to interact with, because the negative charge on the first protonation site makes the second one more difficult to deprotonate.

Calbindin D_{9k}

For calbindin D_{9k} the MEAD method was used to calculate pK_{intr} values and site–site interaction matrix elements for the N- and C-termini and for the side chains of all Asp, Glu, Lys, and Tyr residues. There are no His, Cys, or Arg residues. Unless otherwise noted, the ionic strength was 0.05 M, which corresponds to most

Fig. 8. Cooperativity as a function of $\text{pH} - pK_{\text{intr}}$, displaying anticooperative effects of the pair of anionic protonation sites in the geometry of Figure 7.

of the experiments of (Linse et al., 1991). The cluster method was used to calculate protonation as a function of pH for each titrating group. Such calculations were done for the apo state, the state with Ca^{2+} in binding site I, the state with Ca^{2+} in binding site II, and the doubly Ca^{2+} -loaded state. Because the focus of this study is on a nonconformational mechanism of cooperativity, we used the same protein coordinates (which come from the crystal structure of the doubly loaded state) in the calculations for all of the Ca^{2+} binding states. The only changes we made to the coordinate files to represent changes of Ca^{2+} binding were removals of the Ca^{2+} -ion entries themselves. After the four sets of titration curves were obtained, we used the integral formula (Equation 20) to calculate the cooperativity $\Delta\Delta G_c$ as a function of pH.

Results for the wild-type protein are shown in Figure 9. In the pH range from 1 to 12, the effect of the protonation sites upon cooperativity is to approximately cancel the anticooperative effect of the electrostatic repulsion between the two Ca^{2+} ions. This accounts for over half of the total cooperative influence that is needed to first overcome repulsion and then produce the observed net cooperativity of 7.7 ± 1.5 kJ/mol. There is little difference between the results for ionic strengths of 0.05 and 0.15, for which $-\Delta\Delta G_c$ values of 7.7 ± 1.5 and 4.6 ± 0.9 kJ/mol, respectively, were measured at pH 7.5 (Linse et al., 1991). Although the calculations at higher ionic strength predict a reduction of the anticooperative effect of ligand–ligand repulsion, the cooperative influence of the protonation sites is also reduced, and the two effects cancel.

Examination of the contribution to the integrals in Equation 19, coming from individual protonation sites, shows that the cooperative effects come mostly from six carboxylic acid side chains. The residues bearing these side chains are listed in Table 1, along with their calculated pK_a values in each of the ligand states. Their locations are illustrated in Figure 10. One of these residues is in the first ligation shell of Ca^{2+} -binding site I, and three are in the inner ligand sphere of Ca^{2+} -binding site II (Linse et al., 1988). To our knowledge, no experimentally measured pK_a values for these residues are available.

The very large pK_a shifts seen in Table 1 correspond to the very wide pH dependence seen in Figure 9. These unphysically extreme

Fig. 9. pH dependent cooperativity of wild-type calbindin D_{9k} calculated using Equation 20. Solid curve: 0.05 M ionic strength; dashed curve: 0.15 M ionic strength. The plots extend beyond the pH range in which ionic strength could be held at low values, to show the general trends of the pH dependence.

Table 1. pK_a 's of sites affecting cooperativity

Site	Location ^a	Ligand state			
		00	01	10	11
Asp-19		4.22	1.47	4.48	1.73
Glu-27	I	21.82	−9.42	21.09	−10.10
Asp-54	II	16.88	16.50	−2.89	−3.26
Asp-58	II	9.14	9.80	−0.11	−0.56
Glu-60		13.22	8.08	11.76	8.11
Glu-65	II	12.71	12.56	−8.90	−9.39

^aI or II indicate that the protonation site is located within the inner ligand sphere of ligand-binding site I or II.

shifts result from the combination of strong charge–charge interactions and the assumption of structural rigidity used in these calculations. When a ligand-binding site is occupied by a Ca^{2+} ion, it becomes highly unfavorable to protonate the carboxylate groups in the inner ligand sphere, and the pK_a is pushed to an abnormally low value. One expects that, in reality, protonation of such a carboxylate would be accompanied by a conformational change placing the group in a more favorable position for the protonated state and/or the release of the Ca^{2+} ion. A similar argument applies to the Ca^{2+} -free states. In this case, the binding-site environments (which, in the calculations, are those of the Ca^{2+} -loaded crystal structure) will tend to have a strongly negative electrostatic potential so as to have a high affinity for the positive ion. This tends to raise the calculated pK_a values, but one expects that conformational change upon protonation or deprotonation would moderate this effect.

Fig. 10. Ribbon representation of the backbone of calbindin D_{9k} showing Ca^{2+} binding sites I and II as spheres on the left and right, and the group of acidic side chains involved in cooperativity in ball-and-stick representation.

Several calculations were done to mimic the replacement of one or more protonation sites by nonionizable groups, by removing the corresponding pK_{int} values and site-site coupling matrix elements from the output of the MEAD calculations prior to the titration curve calculations. This amounts to freezing the protonation group in its neutral form. Plots of $\Delta\Delta G_c$ corresponding to the neutralization of Asp-19 and Glu-60 are shown in Figure 11. According to experimental measurements at ionic strength 0.05 and pH 7.5, mutation of Asp19 to Asn reduces the cooperativity from $-\Delta\Delta G_c = 7.7 \pm 1.5$ to 4.0 ± 1.5 kJ/mol, while mutation of Glu60 to Gln reduces it to 3.9 ± 0.9 kJ/mol (Linse et al., 1991). These results are not well reproduced by the present calculations, although for the E60Q mutant in the alkaline pH range, we calculate a reduction of cooperativity of similar magnitude to that seen experimentally at neutral pH. The discrepancies between the calculations and the mutation experiments might occur because mutation changes the conformation in ways not modeled here, or because these residues in the wild-type take part in a conformational mechanism of cooperativity in addition to, or instead of, the electrostatic coupling mechanism considered here.

Neutralization of selected protonation sites can also be used to test ideas about how these sites might influence cooperativity. There are three carboxylic acid groups in the inner ligand shell of site II, all of which have very high calculated pK_a values in the Ca^{2+} -free state and very low pK_a values in the Ca^{2+} -bound state of site II. This means that in the neutral pH range, the binding of Ca^{2+} to site II is calculated to result in the release of two to three protons from the inner ligand shell. If only two protons are released, the situation is analogous to the two-protonation site case corresponding to the $\lambda = 1$ curve of Figure 6A. The Ca^{2+} charge is fully compensated by the release of two protons, so that the other Ca^{2+} site, and perhaps most other protonation sites, do not “see” any net change of the charge. The anticooperative effect of ligand–ligand repulsion is removed, but no net cooperativity results. This suggests that reducing the number of protonation groups in the inner ligand sphere of site II from three to one might allow other protonation groups between the Ca^{2+} -binding sites to come into play and produce a net cooperativity. A test of this idea is shown by the curve in Figure 11 corresponding to the neutralization of the

site II residues, 54 and 58. Net cooperativity does indeed result over part of the pH range, but in other pH ranges, cooperativity is decreased due to the neutralizations. In view of the possibility of anticooperative results presented in the previous subsection on two-protonation-site models, it should not be too surprising that the removal of some protonation sites could increase cooperativity.

Discussion

Electrostatic repulsion between ligands of the same type exerts an anticooperative influence on the binding of charged ligands to proteins, particularly when multiple-binding sites are in the same protein subunit or domain. In this paper, we have shown that electrostatic interactions can also have a cooperative influence when there are sites of protonation/deprotonation that have significant electrostatic interactions with the ligand-binding sites. Cooperative effects of this type will be pH dependent. This mechanism of cooperativity does not require any conformational change, but it could occur in conjunction with conformational changes.

The results presented here would be much the same had a macroscopic definition of cooperativity, such as that of Equation 3, been chosen rather than the microscopic definition, Equation 1. As we pointed out in the presentation of Equation 7, the microscopic cooperativity is independent of the intrinsic binding properties of the ligand-binding sites (the ΔG_a , or alternatively, the microscopic binding constants, K_I and K_{II}). This independence carries all the way through the consideration of coupling to protonation sites and the expression for the pH-dependent cooperativity, Equation 18. The macroscopic cooperativity of Equation 3 becomes identical to the microscopic definition when the intrinsic binding properties of the two ligand sites are identical. Given a system with nonidentical ligand binding sites, one can, as a thought experiment, convert in into a system for which the macroscopic and microscopic cooperativities are identical by altering the intrinsic binding free energies so that $\Delta G_{a,1} = \Delta G_{a,2}$. This has no effect on the microscopic cooperativity, while as shown in Equation 4, it alters the macroscopic cooperativity measure by an amount that depends only on the ratio of the original intrinsic binding constants. Therefore, the total cooperativity, as measured by the macroscopic or microscopic definitions, may differ by a constant for systems with nonequivalent ligand sites, but the pH-dependent contribution coming from electrostatic interactions with protonation sites would be the same.

A study of some simple cases has provided an indication of the magnitude of the effects and their dependence on the distances between the protonation sites and ligand sites. The simplest possible case in which this cooperative mechanism can occur is the case of one protonation site and two ligand-binding sites. In this case, the influence of the protonation site is always cooperative because the binding of the first ligand will tend to shift the equilibrium of the protonation state so as to shift its charge in a direction opposite to the ligand's charge. This makes the binding of the second ligand more favorable. This cooperative mechanism becomes vanishingly small in pH ranges where ligand binding cannot significantly shift the protonation equilibrium. The one-protonation-site case provides an opportunity for a fairly complete study of geometric effects. If the protonation site is located very near one of the ligand-binding sites, its effect will be to cancel $(z - 1)/z$ of the anticooperative effect of ligand–ligand repulsion. If the protonation site is located midway between the two ligand-binding sites, it is possible to get a greater cooperative effect which may produce

Fig. 11. Cooperativity as a function of pH for calbindin D9k with selected residues neutralized. The notation in the legend indicates the residue or residues neutralized. The wild-type (no neutralizations) is shown for reference.

net cooperativity. This is more likely to occur when the magnitude of the ligand charge is smaller, and when the effective dielectric constant for site-site interactions increases with distance.

Model systems with two protonation sites and two ligand-binding sites have more complex behavior, although in many cases they can be understood in terms of analogous one-protonation-site systems. With two protonation sites, it is possible for the effect of the protonation sites to be anticooperative for some combinations of geometry and charge, but such cases are expected to be rare.

Our calculations on the cooperativity of the Ca^{2+} binding of calbindin D_{9k} have shown that electrostatic coupling to protonation sites can make a significant contribution to the cooperative effect in a real protein. We obtain a calculated cooperative effect that cancels the anticooperative effect of Ca^{2+} repulsion. This accounts for over half of the total cooperative effect needed to produce the experimentally observed net cooperativity. The remainder could well come from subtle conformational changes of the kind that have been studied by NMR techniques (Carlström & Chazin, 1993; Akke et al., 1995). The strict exclusion of conformational change from the present calculations is somewhat artificial and probably causes some error, but it allows us to study the contribution of the electrostatic couplings to cooperativity in a "pure" case.

If the effect of conformational change upon these electrostatic interactions and pK_a shifts were better accounted for, what changes in the calculated cooperative behavior might be seen? We believe that the main changes in such calculations are likely to be a reduction of the strength of site-site couplings and more moderate pK_{int} values. In the one- and two-protonation-site calculations, shifts of pK_{int} changed the pH dependence but not the magnitude of the cooperative effects. Reduction of couplings between the protonation sites and the ligand sites should usually reduce the cooperative effects. However, if the largest reductions occur for inner ligand shell interactions, the effects would be like moving from the $\lambda = 1$ curves, in Figure 3, 5A, or 5B, to the curves of less extreme values of λ . Therefore, we suspect that calculations that better accounted for conformational change would give cooperative effects over a more moderate pH range, but that the magnitude of the effects would not be much reduced.

It is likely that the cooperative mechanism described here occurs in other proteins that bind a charged ligand to more than one site within the same protein domain. Protein surfaces are rich in ionizable side chains that undergo protonation and deprotonation over a wide range of pH values. A binding site for a charged ligand will probably be near several such side chains, and some of these protonation sites will probably also have significant electrostatic couplings with more than one of the ligand-binding sites. Thus, the binding of a ligand to one site will have the possibility of shifting the protonation equilibrium in a manner that affects other ligand-binding sites through the electrostatic couplings. The mechanism may even extend to the binding of neutral ligands if ligand binding is coupled to the protonation or deprotonation of groups within the binding sites. Experimentally these effects should be pH-dependent, but if several protonation sites are involved, the pH-dependence may be complex and the cooperative effects may extend over a fairly broad range. The ideal experimental test for the mechanism would be measurements of the uptake or release of protons as the protein goes from one ligand-binding state to another (Saroff, 1993). Ionic strength dependence could also occur, although the calculations on calbindin D_{9k} show that ionic strength effects can cancel out since they reduce both the anticooperative ligand-ligand repulsion and the cooperative ligand-protonation site interactions.

The cooperative mechanism described here could occur along with conformational mechanisms in the same protein. In fact, conformational mechanisms and coupling to protonation sites should be closely interconnected since conformational changes can also be coupled to protonation and deprotonation (Gilson, 1993; You & Bashford, 1995). It is hoped that future studies combining both mechanisms will lead to a better understanding of cooperative effects.

Methods

Coordinates for bovine calbindin D_{9k} are taken from the 1.6 Å resolution X-ray crystallographic model (Svensson et al., 1992), as deposited in the Protein Data Bank (Bernstein et al., 1977) under the file name "4icb." The side-chain coordinates of the N-terminal methionine are absent from this file, so they were placed in a minimum energy conformation using the CHARMM computer program (Brooks et al., 1983). Hydrogen atom positions were generated using the HBUILD procedure (Brünger & Karplus, 1988) as implemented in CHARMM. To relieve any steric clashes present in the X-ray structure, or caused by the building of hydrogen coordinates, the structure was energy minimized with the electrostatic energy term disabled. The minimization protocol was 100 steps of steepest descent minimization followed by conjugate gradient minimization with the stopping criteria that the energy change per step be less than 0.01 kcal/mol. In the above calculations, the CHARMM22 all-hydrogen empirical potential energy function was used (MacKerell et al., 1995).

The pK_{int} and $W_{\mu\nu}$ values for the calculations on calbindin D_{9k} were obtained using the model of macroscopic electrostatics with atomic detail, and the MEAD computer program suite,⁵ which implements this model. These methods have been described in detail (Bashford & Gerwert, 1992). Only a rough sketch is given here. The pK_{int} values are assumed to differ from model compound values by two kinds of electrostatic terms: a Born solvation energy term, which involves the interaction of the titrating charges with the polarization that these charges themselves induce in the surrounding dielectric, and a background term, which involves the interaction of the titrating charges with the background of non-titrating charges in the protein or model compound. The calculation of these terms involves solving the linearized Poisson-Boltzmann equation by the finite difference method for both the protein and the model compound. The $W_{\mu\nu}$ values are obtained from the solutions of the linearized Poisson-Boltzmann equation for the protein. Finite-difference calculations were done using a two-step focusing procedure in which coarse lattice (1.0 Å spacing) calculations with the lattice centered on molecular center were followed by fine lattice (0.25 Å spacing) calculations with the lattice focused on the titrating site. The probe radius used to determine the dielectric boundary was 1.4 Å, and the ion-exclusion radius used to determine the boundary of the electrolyte region was 2.0 Å. Dielectric constants of 4.0 and 80.0 were used for the protein interior and solvent, respectively. The direct use of Equation 10 is not possible because of the combinatoric problem of a large number of sites, so we have used a hybrid method (Yang et al., 1993) in which clusters of closely interacting sites are treated by an explicitly Boltzmann summation, while more weakly inter-

⁵The MEAD computer programs are available through the Internet by anonymous FTP to <ftp.scripps.edu> under the directory, electrostatics, or through the Bashford Web page, <http://www.scripps.edu/bashford>.

acting sites are treated by the mean-field approach (Tanford & Roxby, 1972).

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