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On the Hill Plot of NMR Data for Titration of Protein Residues

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Abstract. The Hill plots of NMR titration data for protein residues disclose more clearly than the usual titration curves the presence of multiple weak perturbations originating from other titratable groups, and should be used whenever the conventional curve fitting is poor. For a quantitative interpretation, we derive here expressions for the Hill equation and the Hill coefficient when the titration of the observed group is perturbed by more than one titratable group. When the generalized Hill equation is fitted to the data, values of the interaction parameters between the observed group and the others are extracted provided that there are no mutual interactions between the latter groups. The method is applied to the titration data of two histidyl residues of L-arginine phosphotransferase (E.C. 2.7.3.3.) in the transition state analogue complex (enzyme-Mg^{2+}-ADP-NO_3^--L-Arg). From the Hill plots, interactions with three titratable groups are disclosed for both residues, and the fitting with the Hill equation reveals that they experience perturbations from the same three groups. Microscopic pK values are obtained for all the involved groups, indicating large changes (up to 3 pH units) upon protonation of the interacting groups. As compared to the conventional fitting procedure, the use and fitting of Hill plots yields from NMR data more information on the neighbourhood of enzyme residues and on the changes intervening therein through the steps involved in the catalysis.

Key words: Proton NMR – Arginine kinase – Histidyl titration – Hill plots – Cooperativity

Introduction

As is well known, the titration of an ionizable group may be followed by NMR through the chemical shift variation of a proton attached to this group, provided that fast exchange conditions are fulfilled (Emsley 1965). From the chemical

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shift value δ_A of the observed proton $-$ δ_A being the weighted average of the chemical shifts δ_{A1} and δ_{A0} of the fully protonated and unprotonated A species respectively $-$ the protonated fraction $\bar{Y}$ of A may be derived and the familiar Hill plot of log $[\bar{Y}/(1-\bar{Y})]$ vs log H (Hill 1910) is obtained from log $[(\delta_A - \delta_{A0})/(\delta_{A1} - \delta_A)]$ vs pH (Markley 1975), H standing for the concentration (or the activity) of the protons in the solution. The apparent Hill coefficient n_H is:

$$n_H = d \log [\bar{Y}/(1-\bar{Y})]/d \log H = d \log [(\delta_A - \delta_{A0})/(\delta_{A1} - \delta_A)]/d \log H . \quad (1)$$

Only positive values of $(\delta_A - \delta_{A0})/(\delta_{A1} - \delta_A)$ are allowed, thus excluding δ_A either larger than δ_{A1} or smaller than δ_{A0}. The chemical shift of "aromatic" protons is now determined with a relative accuracy of $\pm 5.10^{-4}$ or better, and the corresponding Hill plots reveal meaningful details, provided that the titration is completed within the experimental pH range, since accurate values for δ_{A1} and δ_{A0} are needed.

Among the diagnostic tests of homotropic interactions, (Wyman 1948, 1967; Dahlquist 1974; Schwarz 1976), n_H is normally used as an index of cooperativity for the binding of a given ligand on *all possible binding sites,* alike or not; n_H values > 1 are indicative of positive cooperativity, but it has been shown by Wyman that n_H values < 1 are not necessarily indicative of interaction with negative cooperativity, but are also observed when there exists two types of *non-interacting* binding sites, provided that they are not alike. Since NMR allows the separate observation of different proton binding sites, a particular n_H value relates then to a *single type* of protonation site. Thus, though there exists several types of protonation sites in the molecule, the NMR Hill plot for a given titratable residue is a straight line of unit slope in the absence of interactions with other titratable groups, and *any n_H value differing from 1 is indicative of interaction.* In order to make clearer the meaning of NMR n_H values, we derive here expressions for n_H in the case of two interacting titratable groups; the results are extended to perturbations of a given titration by two then by three titratable groups and a general formula is derived for the Hill equation. We then show, taking the example of an actual enzyme complex, what information may be drawn out of Hill plots to disentangle the perturbations arising from several titratable groups.

Interactions Between Two Titratable Groups

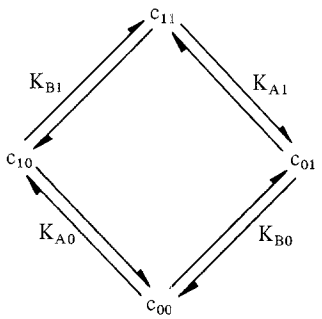
The general mathematical treatment of NMR titration curves for two interacting groups has been developed by Sachs et al. (1971) and by Shrager et al. (1972). From their results[1], expressions for the Hill equation and for the Hill coefficient n_H are obtained as follows.

1 The present derivation is obtained from ionization constants, while these authors have used association constants

Given two titratable groups A and B in a macromolecule, the interaction between A and B may affect either the ionization constants of A and B, or the chemical shifts of the observed protons for the protonated and unprotonated states of A and B, or both. Thus, four different A_{ab} subspecies coexist with concentrations c_{ab}, each subspecies being characterized by a chemical shift δ_{Aab}; here the subscripts a and b relate to the state of protonation of A ($a = 0$ or 1) and of B ($b = 0$ or 1). The ionization constants are K_{A1} or K_{A0} (B protonated or not), and K_{B1} or K_{B0} (A protonated or not). The equilibria between the A subspecies are governed by:

$$c_{00} \cdot H = c_{10} \cdot K_{A0} = c_{01} \cdot K_{B0};\ c_{10} \cdot H = c_{11} \cdot K_{B1};\ c_{01} \cdot H = c_{11} \cdot K_{A1} \qquad (2)$$

as illustrated below



From Eq. (2), it follows that $K_{A0} \cdot K_{B1} = K_{A1} \cdot K_{B0}$. (3)

Since fast exchange is assumed, the observed chemical shift δ_A is the weighted average of the chemical shifts of all the A_{ab} subspecies, i.e.,

$$\delta_A = c_{00}\,\delta_{A00} + c_{01}\,\delta_{A01} + c_{10}\,\delta_{A10} + c_{11}\,\delta_{A11}$$

where $c_{00} + c_{01} + c_{10} + c_{11} = 1$.

I. Interactions Inducing pK and δ Changes

Let $\delta_{A11} - \delta_{A10} = \delta_{A01} - \delta_{A00} = \beta$, $\delta_{A11} - \delta_{A01} = \Delta$, and $\alpha = \beta/(\beta + \Delta)$. The Hill equation in terms of δ's and c's is then:

$$\frac{\delta_A - \delta_{A00}}{\delta_{A11} - \delta_A} = \frac{c_{11}(\delta_{A11} - \delta_{A00}) + c_{10}(\delta_{A10} - \delta_{A00}) + c_{01}(\delta_{A01} - \delta_{A00})}{c_{00}(\delta_{A11} - \delta_{A00}) + c_{01}(\delta_{A11} - \delta_{A01}) + c_{10}(\delta_{A11} - \delta_{A10})}$$

$$\text{or}\quad \frac{\delta_A - \delta_{A00}}{\delta_{A11} - \delta_A} = \frac{c_{11} + c_{10}(1 - \alpha) + c_{01}\alpha}{c_{00} + c_{01}(1 - \alpha) + c_{10}\alpha}. \qquad (4)$$

Using Eq. (2) to express all the concentrations in terms of c_{11}, we get:

$$\frac{\delta_A - \delta_{A00}}{\delta_{A11} - \delta_A} = \frac{H^2 + [K_{B1} + \alpha(K_{A1} - K_{B1})]H}{K_{A1}K_{B0} + [K_{A1} - \alpha(K_{A1} - K_{B1})]H}.$$

Let η and ε be the interaction parameters, η being the true interaction coefficient, i.e., $K_{A1} = \eta K_{A0}$, $K_{B0} = \varepsilon K_{A0}$. From Eq. (3) $K_{B1} = \eta K_{B0}$. Let also $Z = H/K_{A0}$. Then:

$$\frac{\delta_A - \delta_{A00}}{\delta_{A11} - \delta_A} = \frac{Z}{\eta} \cdot \frac{Z + \eta(\varepsilon - \alpha\varepsilon + \alpha)}{Z(1 + \alpha\varepsilon - \alpha) + \varepsilon}. \tag{5}$$

Let $M = \eta(\varepsilon - \alpha\varepsilon + \alpha) = \eta e$, $m = \varepsilon/(1 + \alpha\varepsilon - \alpha)$ and $M/m = \varrho$, ϱ being the apparent interaction coefficient. Then:

$$\frac{\delta_A - \delta_{A00}}{\delta_{A11} - \delta_A} = \frac{mZ}{\varepsilon\eta} \cdot \frac{Z + M}{Z + m}. \tag{6}$$

From Eqs. (1) and (6): $n_H = 1 + \dfrac{(m - M)Z}{(m + Z)(M + Z)} = 1 + \dfrac{m(1 - \varrho)Z}{(m + Z)(\varrho m + Z)}$ (7)

and $dn_H/dZ = [M/(M + Z)^2] - [m/(m + Z)^2]$. (8)

From Eq. (7), one sees that $n_H < 1$ when $\varrho > 1$, and $n_H > 1$ when $\varrho < 1$; $\varrho > 1$ when $\eta > 1$ and $\alpha > 0$, and $\varrho < 1$ when $\eta < 1$ and $\alpha < 0$; but nothing can be predicted if $\eta < 1$ and $\alpha > 0$ or conversely. From Eq. (8), it is clear that the n_H value goes through an extremum $n_{H\,\mathrm{extr}} = 2/(1 + \varrho^{1/2})$ when $Z = Z_{\mathrm{extr}} = \varrho^{1/2} m$ (Eq. 9). For both extremes of the pH range, $n_H = 1$. Indeed, the Hill plot is a sigmoid curve (Wyman 1967) limited by two asymptotes of unit slope and with y-intercepts $y_0 = pK_{A0} - \log(m/\varepsilon\eta)$ when pH $\to 0$, and $y_1 = pK_{A0} - \log(e/\varepsilon)$. Note that the "apparent pK_A" value, corresponding to $\delta_A - \delta_{A00} = \delta_{A11} - \delta_A$, is:

$$pK_{A\,\mathrm{app}} = pK_{A0} - \log[(\varepsilon\eta - mM + Q^{1/2})/2m]$$
$$\text{where } Q = (mM - \varepsilon\eta)^2 + 4m\varepsilon\eta. \tag{10}$$

II. Interactions Inducing only pK Changes

Then $\alpha = 0$, $\eta \neq 1$ and Eq. (5) becomes:

$$(\delta_A - \delta_{A00})/(\delta_{A11} - \delta_A) = (Z/\eta)(Z + \varepsilon\eta)/(Z + \varepsilon). \tag{11}$$

From Eqs. (7) and (8): $n_H = 1 + [\varepsilon(1 - \eta)Z/(\varepsilon\eta + Z)(\varepsilon + Z)]$ (12)

and $n_{H\,\mathrm{extr}} = 2/(1 + \eta^{1/2})$ for $Z_{\mathrm{extr}} = \varepsilon\eta^{1/2}$.

The y-intercepts of the Hill plot are now $y_0 = pK_{A1}$ and $y_1 = pK_{A0}$.

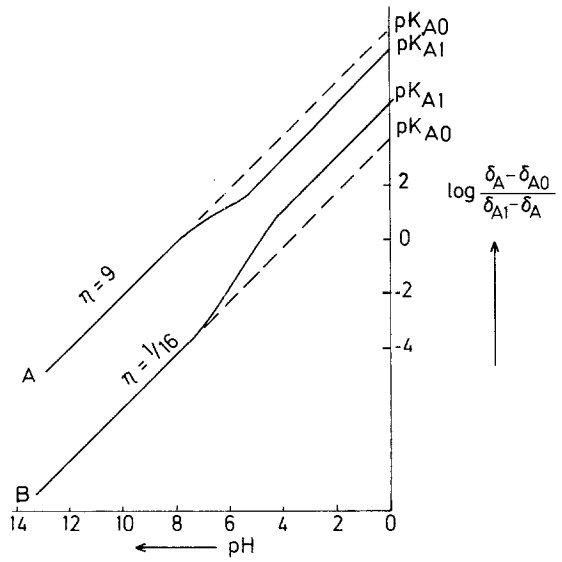


Fig. 1. Schematic plot of log $[(\delta_A - \delta_{A0})/(\delta_{A1} - \delta_A)]$ vs pH calculated for: $\eta = 9$, $pK_{A0} = 5$, $n_{H\,\min}$ is 0.5 (curve A); $\eta = 1/16$, $pK_{A0} = 7$, $n_{H\,\max}$ is 1.6 (curve B)

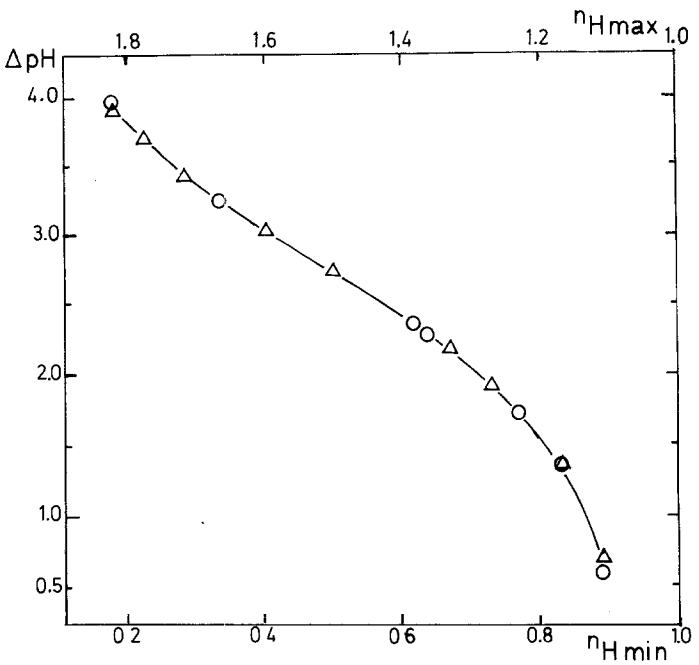


Fig. 2. pH range corresponding to $n_{H\,\min} < n_H < 0.9$ (lower scale) or to $n_{H\,\max} > n_H > 1.1$ (upper scale) plotted respectively versus $n_{H\,\min}$ or $n_{H\,\max}$ (△ or ○)

Examples of theoretical Hill plots are given in Fig. 1 for $\eta = 9$ and $\eta = 1/16$. In Fig. 2 is shown the value ΔpH of the pH range corresponding to n_H varying either between $n_{H\,\text{minimum}}$ and 0.9, or between $n_{H\,\max}$ and 1.1. For the n_H values currently encountered in NMR titrations (0.4–1.6) corresponding to values of η respectively < 16 or > 0.2, the considered pH range is < 3 pH units, and n_H is

Table 1. n_H values from $\log \frac{\bar{Y}}{1-\bar{Y}} = \log \frac{e+Z}{f+Z} \cdot \frac{m}{\varepsilon} Z$, (Model II, see text)

ε		$\varepsilon \ll 1$	$\varepsilon = \frac{\alpha}{\alpha - 1}$	$\varepsilon = 1$	$\varepsilon = \frac{\alpha - 1}{\alpha}$	$\varepsilon \gg 1$
$e = \varepsilon + \alpha(1-\varepsilon)$	$\alpha > 0$			always > 0		
	$\alpha < 0$	< 0		> 0	> 0	
$m = \varepsilon/(1-\alpha-\alpha\varepsilon)$	$\alpha > 0$			always > 0		
	$\alpha < 0$	> 0		> 0	< 0	
$\frac{\bar{Y}}{1-\bar{Y}}$	$\alpha > 0$			always > 0		
	$\alpha < 0$	> 0 when $Z > -e$		> 0	> 0 when $Z < -m$	
n_H	$\alpha > 0$			always < 1		
	$\alpha < 0$	defined only when $Z - e; > 1$		always > 1	defined only when $Z < -m; > 1$	

far from being constant throughout an experimental pH range of 5–6 pH units or more. The fitting of data with a constant value for n_H in such a pH range appears unjustified, though it is currently done.

III. Interactions Inducing only Chemical Shift Changes

Then $\alpha \neq 0$, $\eta = 1$ so that Eq. (5) becomes:

$$(\delta_A - \delta_{A00})/(\delta_{A11} - \delta_A) = (mZ/\varepsilon)\,(Z + e)/(Z + m) \tag{13}$$

$$n_H = 1 + [(m - e)Z/(e + Z)\,(m + Z)]\,. \tag{14}$$

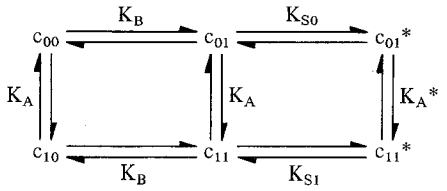
If we define the interaction coefficient η' as $\eta' = e/m$, then

$$n_{H\,\mathrm{extr}} = 2/(1 + \eta'^{1/2}) \text{ when } Z_{\mathrm{extr}} = m\eta'^{1/2}$$

so that $n_H < 1$ when $\eta' > 1$, and since $\eta' = \alpha(1-\alpha)\,(1-\varepsilon)^2/\varepsilon$, when $\alpha > 0$, as α is always < 1. When $\alpha < 0$, the limitations are indicated in Table 1.

IV. Conformation Changes

Conformation changes have been invoked (Tipton and Dixon 1979)[2] to explain the occurrence of n_H values > 1. Such a situation is illustrated below:



2 As pointed out by these authors, positive cooperativity would also occur if the ionization of B facilitated the binding of a multivalent positive ion whose higher charge would repel the proton on A. Such a situation was not encountered in the present studies

where c_{01^*} and c_{11^*} stand for the concentrations of the A subspecies A_{01^*} and A_{11^*} resulting from the conformation change, and $c_{11} = K_{S1}\, c_{11^*}$; this implies that $K_A \cdot K_{S1} = K_{S0} \cdot K_{A^*}$, and then that the equilibrium for the conformation change depends upon the protonation state of A. The observed chemical shift δ_A is:

$$\delta_A = c_{00}\, \delta_{A00} + c_{01}\, \delta_{A01} + c_{11}\, \delta_{A11} + c_{01^*}\, \delta_{A01^*} + c_{11^*}\, \delta_{A11^*} + c_{10}\, \delta_{A10}\,.$$

If we assume that the perturbations do not affect the chemical shifts, we get for the Hill equation

$$\frac{\delta_A - \delta_{A0}}{\delta_{A1} - \delta_A} = \frac{[\varepsilon K_{S1}/(K_{S1} + 1)] + Z}{[\varepsilon K_{S1}/(K_{S1} + \chi)] + Z} \cdot \frac{K_{S1} + 1}{K_{S1} + \chi} \cdot Z\,, \tag{15}$$

where $K_B = \varepsilon K_A$, $K_{A^*} = \chi K_A$, and $Z = H/K_A$. The relation Eq. (15) is obviously the same as Eq. (6) if $M = \varepsilon K_{S1}/(K_{S1} + 1)$ and $m = \varepsilon K_{S1}/(K_{S1} + \chi)$.

Thus, the Hill equation for the NMR titration of a group A whenever another titratable group B is interacting with A is expressed by the relation Eq. (6), and n_H values may be either < 1 or > 1, indicating apparent negative or positive cooperativity. The result of direct charge interaction involving only pK changes (model II) shows obviously negative cooperativity; the effect is short ranged (since it is proportional to d^{-2}, d being the distance between the two charges assumed to be punctual), isotropic around A and around B and exactly reciprocal, i.e., the pK_A change equals the pK_B change. If the effect of direct charge interaction is to perturb only the extreme chemical shifts, negative cooperativity occurs when $\alpha > 0$, which corresponds to an increased electron density on the A proton when B is ionized and conversely.

If the ionization of B triggers a conformation change which results in a pK_A change (model IV), n_H may be > 1 or < 1, and the effect needs not to be reciprocal.

Another event accompanying the ionization of the B group may be the movement of an aromatic residue previously held in place by hydrogen bonding. A typical example is the ionization of a tyrosyl residue. As the magnetic field generated by the circulating π-electrons is highly *anisotropic,* the movement of the ring may result in an increase or a decrease of the local magnetic field at A, (Bovey 1969), corresponding to the model III with $\alpha < 0$ or > 0; hence, the same residue may interact with two titratable neighbouring groups with $n_H > 1$ for the first and $n_H < 1$ for the other. This effect falls off rapidly with the distance.

From the titration of only one residue, and from the fitting of the data to Eq. (6), we get in the most general case three relations to determine four unknowns. The problem can only be solved if another assumption is made, either $\alpha = 0$ (model of Sect. II, referred to as model II), or $\eta = 1$ (model III), unless an arbitrary value is given to α. In the course of our experimental work, we have observed on the titration curves of histidyl residues mostly pK changes when different ligands were bound on the protein, while the values of the extreme chemical shifts for a given residue remained nearly constant. This justifies the

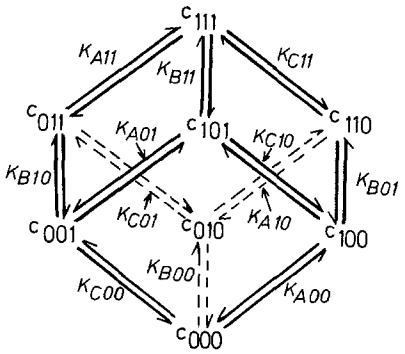


Fig. 3. Equilibria between the different A_{abc} subspecies when the titration of A is perturbed by the titration of B and C; a, b, $c = 1$ or 0 when the corresponding groups, A, B, C are protonated or not; the concentrations of the different A subspecies are c_{abc} and the equilibrium constants K_{Abc}, K_{Bca}, and K_{Cab}

assumption that will be made in order to proceed further, i.e., that any interaction induces mainly pK changes, and model II will be used throughout.

Interactions with More Than One Titratable Group. To take into account the influence of multiple weak perturbations, the model of Sect. II is extended to the perturbation of the A titration by the titration of two groups B and C, then to the perturbation by three groups B, C, and D.

Interaction of A with B and C. The different steps between A_{111} (A protonated in the presence of B and C protonated) and A_{000} (all three groups unprotonated) are depicted in Fig. 3 by an array of the eight possible A subspecies and of the twelve possible ionization equilibria. We define c_{abc} as the concentration of a particular A subspecies for which a, b, c are equal either to one or to zero depending upon whether A, B, and C are protonated or not. The ionization constants relating c_{0bc}, c_{A0c}, c_{ab0} to c_{abc} are respectively K_{Abc}, K_{Bca}, and K_{Cab}. For example,

$$c_{111}/H = c_{011}/K_{A11} = c_{101} \cdot K_{B11} = c_{110} \cdot K_{C11}$$
$$\text{and } c_{001} \cdot H = c_{011} \cdot K_{B10} = c_{101} \cdot K_{A01} .$$

The corresponding chemical shifts are δ_{Aabc}. Since we assume no chemical shift perturbations:

$$(\delta_A - \delta_{A0})/(\delta_{A1} - \delta_A) = (c_{111} + c_{110} + c_{101} + c_{100})/(c_{000} + c_{001} + c_{010} + c_{011}) . \quad (16)$$

From the mass law: $c_{0bc} = c_{abc} K_{Abc}$, etc., and as there exists between the different equilibrium constants a set of relations such as:

$$K_{A11} K_{B10} K_{C00} = K_{B11} K_{C10} K_{A00} = K_{C11} K_{A10} K_{B00},$$
$$K_{A11} K_{B10} = K_{B11} K_{A01} \text{ etc.}$$

all the concentrations may be expressed in terms of only one, e.g., c_{111}, and

$$\frac{\delta_A - \delta_{A0}}{\delta_{A1} - \delta_A} = \frac{H^2 + (K_{B11} + K_{C11})H + K_{B11} \cdot K_{C10}}{H^2 + (K_{C01} + K_{B10})H + K_{C00} \cdot K_{B10}} \cdot \frac{H}{K_{A11}} . \tag{17}$$

Setting

$$K_{B00} = \varepsilon_1 K_{A00}, \; K_{B01} = \eta_1 K_{B00}, \; K_{A10} = \eta_1 K_{A00} ,$$

$$K_{C00} = \varepsilon_2 K_{A00}, \; K_{C10} = \eta_2 K_{C00}, \; K_{A01} = \eta_2 K_{A00} ,$$

and defining the mutual $B-C$ interaction as

$$K_{C01} = \mu K_{C00} \text{ and } K_{B10} = \mu K_{B00}$$

we express all the equilibrium constants in terms of K_{A00} and use the reduced variable $Z = H/K_{A00}$, and we get:

$$\frac{\delta_A - \delta_{A0}}{\delta_{A1} - \delta_A} = \frac{Z}{\eta_1 \eta_2} \cdot \frac{Z^2 + (\eta_1 \varepsilon_1 + \eta_2 \varepsilon_2)\mu Z + \eta_1 \varepsilon_1 \eta_2 \varepsilon_2 \mu}{Z^2 + (\varepsilon_1 + \varepsilon_2)\mu Z + \varepsilon_1 \varepsilon_2 \mu}$$

$$= \frac{Z}{\eta_1 \eta_2} \cdot \frac{R}{S} . \tag{18}$$

If R and S are factorable, then

$$R = (Z + M_1)(Z + M_2) ; \quad S = (Z + m_1)(Z + m_2) \text{ and}$$
$$M_1 + M_2 = (\eta_1 \varepsilon_1 + \eta_2 \varepsilon_2)\mu ; \quad M_1 M_2 = \eta_1 \varepsilon_1 \eta_2 \varepsilon_2 \mu . \tag{19}$$

$$m_1 + m_2 = (\varepsilon_1 + \varepsilon_2)\mu ; \quad m_1 m_2 = \varepsilon_1 \varepsilon_2 \mu . \tag{20}$$

Eqs. (19) and (20) define two quadratic equations and the conditions for real roots are

$$(\eta_1 \varepsilon_1 + \eta_2 \varepsilon_2)^2 - 4 \varepsilon_1 \varepsilon_2 \eta_1 \eta_2 / \mu \geqslant 0$$

and

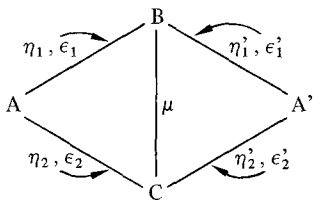
$$(\varepsilon_1 + \varepsilon_2)^2 - 4 \varepsilon_1 \varepsilon_2 / \mu \geqslant 0 .$$

If it happened that μ were small enough to make one of the above quantities negative, which would correspond to an interaction with strong positive cooperativity (since it is readily seen that this corresponds to $\mu < \frac{1}{2}$) then the factorization would be impossible. In all other cases, the experimental data may be fitted with the function

$$\log [(\delta_A - \delta_{A0})/(\delta_{A1} - \delta_A)] = \log Z + \log [(Z + M_1)/(Z + m_1)]$$
$$+ \log [(Z + M_2)/(Z + m_2)] + \text{constant} . \tag{21}$$

As any of the models I, II and III leads to the same general Eq. (6), the factorization is always possible, with the above restriction regarding μ. In the frame of model II when $\mu = 1$, then m_1, m_2, M_1/m_1, and M_2/m_2 are respectively identical with ε_1, ε_2, η_1, and η_2. If $\mu \neq 1$, the true ε's and η's will not be accessible from the sole titration of one residue A perturbed by two other titratable groups B and C.

Nevertheless, when the observation of two titratable groups A and A' reveals two perturbations on each, one may ask if they originate from the same B and C groups, or if A' is one of the groups perturbing A and conversely. In the first case – implying that A and A' do not interact – let $K_{A'00}$, ε'_1, η'_1, etc., be the relevant parameters for the A' group. The interactions are shown on the following diagram:



Let $K_{A'00} = \gamma K_{A00}$. The ionization constant of B is perturbed by the ionization of A, A', and C, and three subscripts are now required to identify K_B, i.e. $K_{Bcaa'}$. Either $K_{B001} = \varepsilon_1 K_{A00}$ or $K_{B000} = \varepsilon_1 K_{A00}$ depending if the adjustment made on the A titration yields K_{B00} for A' protonated or not. In the first case, that is when

$\gamma \gg 1$, as $K_{B001} = \eta'_1 K_{B000}$, then $\gamma = \varepsilon_1/\eta'_1 \varepsilon'_1$; and also $\gamma = \varepsilon_2/\eta'_2 \varepsilon'_2$, so that

$$(m_1 + m_2)/(M'_1 + M'_2) = \gamma \text{ and } m_1 m_2/M'_1 M'_2 = \gamma^2. \tag{22}$$

Similarly, when

$$\gamma \ll 1 \quad (m'_1 + m'_2)/(M_1 + M_2) = \gamma \text{ and } m'_1 m'_2/M_1 M_2 = \gamma^2 . \tag{23}$$

If γ does not differ much from 1 (for instance $2 > \gamma > 0.5$), then

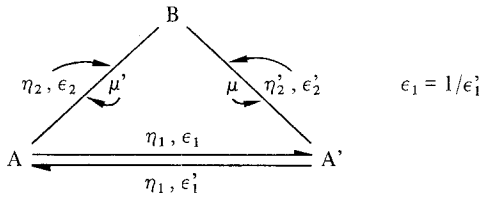
$$(m_1 + m_2)/(m'_1 + m'_2) = \gamma \text{ and } m_1 m_2/m'_1 m'_2 = \gamma^2 . \tag{24}$$

Thus, when the model II is likely to apply, whenever one of these sets of relations will be found to exist and yield a ratio equal to $\gamma = K_{A'00}/K_{A00}$, we have proof that A and A' are perturbed by the same B and C groups; but μ remains undetermined.

If one of the interactions, $A-C$ for instance, occurs via a conformation change, in the conditions of the model IV, then $m_1 = \varepsilon_1$, $M_1 = \varepsilon_1 \eta_1$, $m_2 = \varepsilon_2 K_{S1} / (\chi + K_{S1})$, and $M_2 = \varepsilon_2 K_{S1}/(1 + K_{S1})$; K_{S1} and K_{S0} are the equilibrium constants for the conformation change triggered by the protonation of C when A is protonated and unprotonated respectively and $K_{S1} = \chi K_{S0}$. If the

same C group interacts also with A' ($K_{A'0} = \gamma K_{A0}$ and $\gamma \simeq 1$) then K_{S0} must have the same value as "seen" from A and from A'. Hence, the same relations Eq. (24) still hold.

If A' is suspected to be one of the two titratable groups perturbing A and conversely, the situation is as follows:



From the fitting of the A titration (m_1, m_2, M_1, M_2, K_{A00}) a value for μ may be drawn (since ε_1 is known from the fitting of the A' titration as $K_{A'00} = \varepsilon_1 K_{A00}$), using Eq. (20), i.e.,

$$\mu = [\varepsilon_1(m_1 + m_2) - 2\, m_1\, m_2]/2\, \varepsilon_1^2\,, \tag{25}$$

μ being known, values for η_1, η_2, and ε_2 may be obtained. Similarly, from the fitting of the A' titration, μ', η_1, η_2', and ε_2' are obtained. If the same value for η_1 is afforded by both adjustments and if $\mu = \eta_2'$ and $\mu' = \eta_2$, we then have proof that A and A' are mutually interacting and that they also interact with the same group B.

More generally, if J titratable groups ($J = B, C, D, \ldots$) with equilibrium constants $K_{J0} = \varepsilon_j K_{A0}$ and $K_{J1} = \eta_j K_{J0}$ ($j = 1, 2, 3, \ldots$) are interacting with the observed A group, if there is no mutual interaction between the perturbing groups (μ's = 1) a generalized form of Eq. (6):

$$\log [(\delta_A - \delta_{A0})/(\delta_{A1} - \delta_A)] = \log Z + \sum_j \log [(M_j + Z)/(m_j + Z)] + \text{constant} \tag{26}$$

is a suitable representation of the titration data. If μ's $\neq 1$, we derive now an expression for the interaction of A with three groups B, C, D assuming the validity of the model II (pK changes only).

Interaction of A with B, C, D. To the previous parameters, we add the interaction parameters between A and D, i.e., η_3 and ε_3, and the interaction coefficients between B and C (μ_1), C and D (μ_2), D and B (μ_3). The equilibrium constants are now identified by four subscripts: K_{Abcd} K_{Bcda}, K_{Cdab} and K_{Dabc}, and the concentrations of the A subspecies by c_{abcd}. Any protonation equilibrium is represented by

$$c_{abc0} \cdot H = c_{abcd} \cdot K_{Dabc} \tag{27}$$

and sets of relations such as

$$\begin{aligned} K_{D111} \cdot K_{C011} &= K_{C111} \cdot K_{D110} \\ K_{D111} \cdot K_{C011} \cdot K_{B001} &= K_{B111} \cdot K_{C110} \cdot K_{D100} \\ K_{D111} \cdot K_{C011} \cdot K_{B001} \cdot K_{A000} &= K_{A111} \cdot K_{C101} \cdot K_{D010} \cdot K_{B000} \end{aligned} \tag{28}$$

exist between the different equilibrium constants.

From the extension of the model of Sect. II, we get

$$\frac{\delta_A - \delta_{A0}}{\delta_{A1} - \delta_A} = \frac{Z}{\eta_1 \eta_2 \eta_3} \cdot \frac{Z^3 + UZ^2 + VZ + W}{Z^3 + uZ^2 + vZ + w}, \tag{29}$$

where:

$$\begin{aligned} U &= [(\varepsilon_1 \eta_1/\mu_3) + (\varepsilon_2 \eta_2/\mu_2) + (\varepsilon_3 \eta_3/\mu_1)]\ \mu_1 \mu_2 \mu_3 \\ V &= (\varepsilon_1 \eta_1 \varepsilon_2 \eta_2 + \varepsilon_2 \eta_2 \varepsilon_3 \eta_3 + \varepsilon_3 \eta_3 \varepsilon_1 \eta_1)\ \mu_1 \mu_2 \mu_3 \\ W &= \varepsilon_1 \eta_1 \varepsilon_2 \eta_2 \varepsilon_3 \eta_3 \mu_1 \mu_2 \mu_3 , \end{aligned} \tag{30}$$

$$\begin{aligned} u &= [(\varepsilon_1/\mu_3) + (\varepsilon_2/\mu_2) + (\varepsilon_3/\mu_1)]\ \mu_1 \mu_2 \mu_3 \\ v &= (\varepsilon_1 \varepsilon_2 + \varepsilon_2 \varepsilon_3 + \varepsilon_3 \varepsilon_1)\ \mu_1 \mu_2 \mu_3 \\ w &= \varepsilon_1 \varepsilon_2 \varepsilon_3 \mu_1 \mu_2 \mu_3 . \end{aligned} \tag{31}$$

Both polynoms of 3rd degree in Z which appear in Eq. (29) are factorable if they can be identified respectively with $(Z + M_1)\ (Z + M_2)\ (Z + M_3)$ and $(Z + m_1)\ (Z + m_2)\ (Z + m_3)$ where

$$\begin{aligned} m_1 + m_2 + m_3 &= u \\ m_1 m_2 + m_2 m_3 + m_3 m_1 &= v \\ m_1 m_2 m_3 &= w \end{aligned}$$

and

$$\begin{aligned} M_1 + M_2 + M_3 &= U \\ M_1 M_2 + M_2 M_3 + M_3 M_1 &= V \\ M_1 M_2 M_3 &= W \end{aligned} \tag{32}$$

m and M are thus roots of the cubic equations:

$$\begin{aligned} m^3 - um^3 + vm - w &= 0 \\ M^3 - UM^3 + VM - W &= 0 . \end{aligned} \tag{33}$$

The existence of three real roots relies upon the conditions:

$$\begin{aligned} (2\ u^3 - 9\ uv + 27\ w)^2 + 4(3\ v - u^2)^3 &< 0 \\ (2\ U^3 - 9\ UV + 27\ W)^2 + 4(3\ V - U^2)^3 &< 0 \end{aligned}$$

which are verified for values of μ_1, μ_2, μ_3 close to 1 (weak mutual interactions between B, C, D).

Table 2. Relations between apparent parameters M's and m's for two groups A and A′ perturbed by the same three groups B, C, D (Model II, μ's $\neq 1$)

	$\gamma \simeq 1$	$\gamma \gg 1$	$\gamma \ll 1$
$\varepsilon_1/\varepsilon_1' =$	γ	$\eta_1' \gamma$	γ/η_1
$\varepsilon_2/\varepsilon_2' =$	γ	$\eta_2' \gamma$	γ/η_2
$\varepsilon_3/\varepsilon_3' =$	γ	$\eta_3' \gamma$	γ/η_3
$\gamma =$	$\frac{m_1 + m_2 + m_3}{m_1' + m_2' + m_3'}$	$\frac{m_1 + m_2 + m_3}{M_1' + M_2' + M_3'}$	$\frac{m_1' + m_2' + m_3'}{M_1 + M_2 + M_3}$
$\gamma^2 =$	$\frac{m_1m_2 + m_2m_3 + m_3m_1}{m_1'm_2' + m_2'm_3' + m_3'm_1'}$	$\frac{m_1m_2 + m_2m_3 + m_3m_1}{M_1'M_2' + M_2'M_3' + M_3'M_1'}$	$\frac{m_1'm_2' + m_2'm_3' + m_3'm_1'}{M_1M_2 + M_2M_3 + M_3M_1}$
$\gamma^3 =$	$\frac{m_1m_2m_3}{m_1'm_2'm_3'}$	$\frac{m_1m_2m_3}{M_1'M_2'M_3'}$	$\frac{m_1'm_2'm_3'}{M_1M_2M_3}$

If the interactions can be represented by the model III or I, provided that all μ's are close to 1, the titration data may also be fitted to the ratio of two factorable polynoms, but from the m and M values so obtained, the true interaction parameters will not in general be accessible.

If μ_i values are unknown, more information may nevertheless be gained from the comparison of the apparent parameters obtained for two titratable residues A and A' when the same number of perturbations are detected on both Hill plots. Let $K_{A'000} = \gamma K_{A000}$, so that $Z' = Z/\gamma$. From the adjustments, values of m_i, M_i (for A) and m_i', M_i' (for A') are available. We want to check if the same three groups are perturbing A and A'; if so, $\mu_1 = \mu_1'$, $\mu_2 = \mu_2'$, $\mu_3 = \mu_3'$. Moreover, the same values for K_B, K_C, K_D when *all* other species are unprotonated must be obtained from the data pertaining to A and from those pertaining to A'. In Table 2 are given, for γ either close to 1, or much smaller or larger than 1, the relations between corresponding ε_i and ε_i' values and the γ value, together with the relations expected to hold between γ and the apparent parameters obtained from fitting with Eq. (26). If, for the proper γ range, the corresponding set of relations is verified by the apparent parameters, and if the values obtained for the ratios compare equally well with the γ value obtained from $K_{A'000}/K_{A000}$, then it may be assessed that the same three B, C and D groups are perturbing both A and A'. Obviously, when all μ's = 1, the same hypothesis leads to a constant value equal to γ for each ratio m_i/M_i', or m_i'/M_i, or m_i/m_i' depending if the γ value is respectively $\gg 1$, $\ll 1$ or $\simeq 1$.

Results and Discussion

We have tested the applicability of Eq. (27) to the NMR titration of two histidyl residues (hereafter referred to as His 1 and His 2) of the arginine kinase molecule in the transition state analogue complex [enzyme-Mg^{2+}-ADP-NO_3^--L-Arg] where the planar ion NO_3^- mimics the transferred phosphoryl in its planar transitory form (Milner-White and Watts 1971). It has been

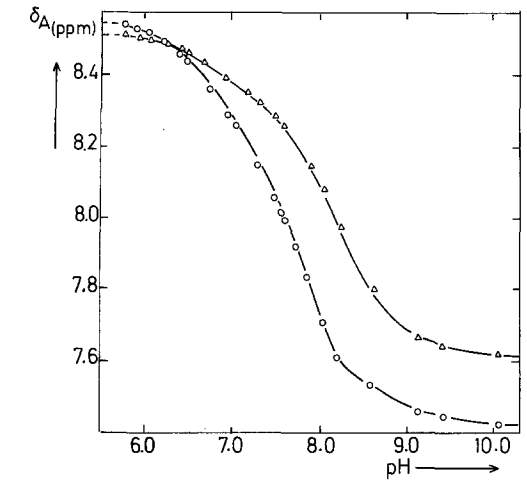


Fig. 4. Titration curves for histidyl residues His 1 (△) and His 2 (○) of arginine kinase in the transition complex APK-Mg^{2+}-ADP-NO_3^--L-Arg. Concentrations: 1 mM APK, 1.2 mM other components in D_2O/50 mM Tris. Each point was obtained at 10° C, NMR at 250 MHz, Fourier mode, 200–400 scans

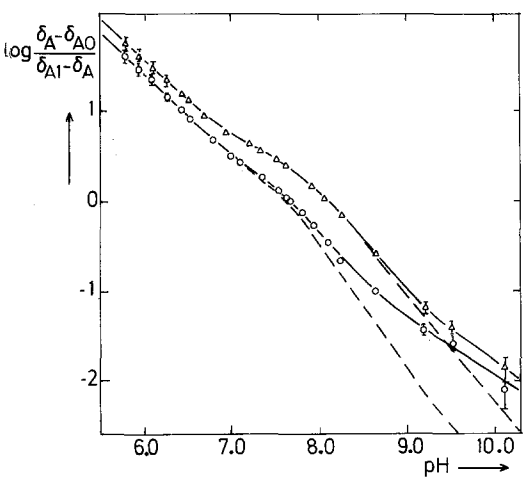


Fig. 5. Hill plots for the titrations of Fig. 4. His 1 (△) and His 2 (○). The dotted lines are calculated (see text) assuming two perturbations with $G_1 = 16$, $g_1 = 1$, $g_2 = 1{,}75$, $G_2/g_2 = 0.1$ (His 1) and with $G_1' = 6$, $g_1' = 1$, $G_2'/g_2' = 0.05$, $g_2' = 1.5$ (His 2). For the solid lines, an additional perturbation has been assumed with $g_3 = 5$, $G_3/g_3 = 0.01$ (His 1), and $G_3'/g_3' = 22$, and $g_3' = 0.01$ (His 2). Error bars are indicated for each experimental point

demonstrated that one histidyl residue of arginine kinase is essential for catalytic activity (Roustan et al. 1970). Indeed, as we have seen on the NMR spectra of ethoxyformylated arginine kinase it is His 1 which appears mainly affected by the chemical modification. The titration curves shown in Fig. 4 were obtained at 250 MHz (Fourier mode), at 10° C for a concentration of 1 mM for the enzyme, and 1.2 mM for each other component of the complex; the solutions were made up in D_2O/Tris 50 mM, and the pH was adjusted with either NaOD or CD_3COOD. The pH values are uncorrected pH-meter reading values. Each point of the titration curve was obtained with about 400 scans. As seen in Fig. 4, no large perturbation of the conventional titration curves is evident; nevertheless, when these curves were fitted to Shrager's models, and even when the standard error was less than the experimental uncertainty, "long runs of positive errors followed by long runs of negative errors" (Shrager) were obtained from least square adjustments. As shown by the Hill plots (Fig. 5), the reasons were

perturbations by three titratable groups for each histidyl, whereas the usual fitting had been of no help in disclosing, and moreover characterizing such interactions. The similarities of the Hill plots for both histidyls are striking. The fitting of both plots to Eq. (26) has been performed in assuming the validity of the model II, and starting from approximate values of $n_{H\,\mathrm{extr}}$, then of η_i's drawn directly out of the Hill plots. Then, starting from the left (or right) part of the Hill plot, two sets of parameters G's and g's are adjusted, setting arbitrarily $g_1 = 1$. When the concordance is obtained with this part of the curve (Fig. 5, interrupted line), a third perturbation is added and all g's and G's are varied until the best fit is arrived at; the quality of the adjustment is checked by superposition of the calculated and experimental Hill plots. As all the parameters have been obtained using an arbitrary variable $X = H/K_N$, we need now, setting $Z = \nu X$, i.e., $K_N = \nu K_{A000}$, to determine ν.

$$\text{When } X = 1\,,\ q_1 = \log\left[(\delta_A - \delta_{A0})/(\delta_{A1} - \delta_{A0})\right]$$

$$= \log \nu + \sum_{j=1}^{3} \log\left[(G_j + 1)/(g_j + 1)\right] - \sum_{j=1}^{3} \log\,(G_j/g_j)\,.$$

The ordinate q_1 of the experimental Hill plot corresponding to $X = 1$ is then read and thus ν is obtained.

The adjustment represented in Fig. 5 for each histidyl residue (solid line) has led to the following parameters:

for His 1
$pK_N = 8.1 \pm 0.1$; $\log \nu = 0.20$; $\nu = 1.95$
$pK_{A000} = 8.4 \pm 0.2$
$m_1 = 2$; $M_1/m_1 = 16 \pm 1.5$
$m_2 = 3.5 \pm 0.4$; $M_2/m_2 = 0.1 \pm 0.01$
$m_3 = 0.02 \pm 0.002$; $M_3/m_3 = 5 \pm 0.5$

for His 2
$pK_{N'} = 8.1 \pm 0.1$; $\log \nu' = 0.12$; $\nu' = 1.32$
$pK_{A'000} = 8.2 \pm 0.2$
$m_1' = 1.3$; $M_1'/m_1' = 6 \pm 0.6$
$m_2' = 2 \pm 0.2$; $M_2'/m_2' = 0.05 \pm 0.005$
$m_3' = 0.015 \pm 0.0015$; $M_3'/m_3' = 22 \pm 2$

Since $K_{A'000}/K_{A000} = \gamma = 1.6$, it may be checked if the corresponding set of relations in Table 2 is verified. The following numerical values are obtained: 1.65; 2.7; 3.6; and are respectively compared with the values of γ (1.6), γ^2 (2.56), and γ^3 (4.1): the agreement is satisfactory. It may then be concluded that the same three groups B, C, and D are perturbing both His 1 and His 2. As m_1/m_1', m_2/m_2', and m_3/m_3', respectively equal to 1.5, 1.7, and 1.3, are close to the γ value, the μ values do not differ much from 1; to quantify the effects of the interacting groups, we then assume all μ's = 1. Microscopic pK values and interaction coefficients η_i are easily obtained for the two interactions with negative

Table 3. n_H and pK values for two histidyl residues of arginine kinase and their perturbing groups (see text)

His 1 (A)				His 2 (A′)		
$pK_{A\,app}$	$= 8.12$	± 0.04		$pK_{A'\,app}$	$= 7.65$	± 0.04
pK_{A000}	$= 8.4$	± 0.2	$pK_{B00} = 8.1 \pm 0.3$	$pK_{A'000}$	$= 8.2$	± 0.2
η_1	$= 16$	± 1.5	$pK_{B10} = 6.9 \pm 0.3$	η'_1	$= 6$	± 0.6
$n_{H(A-B)min}$	$= 0.4$	± 0.02	$pK_{B01} = 7.3 \pm 0.3$	$n_{H(A'-B)min}$	$= 0.6$	± 0.03
pK_{A100}	$= 7.2$	± 0.3	$pK_{B11} = 6.1 \pm 0.3$	$pK_{A'100}$	$= 7.4$	± 0.3
χ	$\leqslant 0.1$			χ'	$\leqslant 0.05$	
$n_{H(A-C)max}$	$= 1.5$	± 0.07	$pK_C \geqslant 9$	$n_{H(A'-C)max}$	$= 1.6$	± 0.08
pK_{A010}	$\geqslant 9.4$			$pK_{A'010}$	$\geqslant 9.5$	
pK_{A110}	$\geqslant 8.2$			$pK_{A'110}$	$\geqslant 8.7$	
η_3	$= 5$	± 0.5	$pK_{D00} = 10.1 \pm 0.3$	η'_3	$= 22$	± 2
$n_{H(A-D)min}$	$= 0.6$	± 0.03	$pK_{D10} = 9.4 \pm 0.3$	$n_{H(A'-D)min}$	$= 0.35$	± 0.02
pK_{A001}	$= 7.7$	± 0.3	$pK_{D01} = 8.7 \pm 0.3$	$pK_{A'001}$	$= 6.8$	± 0.3
pK_{A011}	$\geqslant 8.7$		$pK_{D11} = 8.0 \pm 0.3$	$pK_{A'011}$	$\geqslant 8.1$	
pK_{A101}	$= 6.5$	± 0.3		$pK_{A'101}$	$= 6.1$	± 0.3
PK_{A111}	$\geqslant 7.5$			$pK_{A'111}$	$\geqslant 7.4$	

cooperativity (B and D groups). The interaction with positive cooperativity deserves further attention, since it could originate either from a cycle effect or from a conformation change. Since $M_2/m_2 \simeq M'_2/m'_2$, which means that the importance of the perturbation is nearly the same for both residues, a cycle effect known to be anisotropic and rapidly varying with the distance, is highly improbable. Consequently, we conclude that the perturbation from C occurs via a conformation change. As the equilibrium constant K_{S0} must be < 1 for the new conformation to be stable, and as $\chi < 1$, K_{S1} is also < 1. Assuming $K_{S1} = 10^{-1}$, we set lower limits for ε_2 and ε'_2 respectively equal to 3.5 and 4, so that the lower limit for K_C is about 9. In Table 3 are listed the pK values for A and A' with subscripts b, c, d where b, c, $d = 1$ or 0 depending if the corresponding B, C, D groups are protonated or not, and the pK values for B, C, D with subscript a, a' where a, $a' = 1$ or 0 for A and A' protonated or not.

Thus, from the Hill plot of the titration data and the fitting with a calculated curve using Eq. (26), we have obtained evidence that three perturbations affect each histidyl residue, and that they originate from the same three titratable groups. Thus, His 1 and His 2 must be spatially close to each other. Two groups, B and D, are interacting with negative cooperativity while the interaction with the C group occurs through a conformation change. B acts more efficiently on A than on A', suggesting that it is closer to His 1, while D seems closer to His 2. As seen in Table 3, B must be a neutral group, while C and D are basic groups. The pK_B and pK_D values may vary by up to 2 pH units depending on the state of protonation of His 1 and His 2. The present coefficients of interaction would be of more value if they could be put in parallel with X-ray cristallography data allowing both the identification of the perturbing groups and an estimation of their distances to the observed histidyl residues.

However, the interactions evaluated in terms of changes in affinity constants for the protons show clearly that, depending upon the state of protonation of the

interacting residues, the affinity constant of a given residue for protons may be altered by up to three orders of magnitude. Such changes are most efficient in allowing the transfer of protons at lower energy cost, and specially of the proton involved in the catalysis.

The present method of analysis of titration data is of great use whenever the fitting to conventional titration curves proves very poor as a result of multiple weak interactions with neighbouring titratable groups. Though it is less precise that the conventional least square fitting process, its main advantage is that it is able to evidence multiple weak perturbations which would otherwise be undetected. Moreover, the sign of the cooperativity – positive or negative – is clearly apparent from the Hill plots. The fitting of data with the general relation derived here enables one to deal with more than one perturbation, and even with overlapping perturbations from perturbing groups with similar pK values. The parameters which are extracted may lead to the microscopic pK values of all the interacting groups and to the pK changes induced by the loss or gain of proton on any interacting group. Thus, either by disclosing the proximity of titratable groups from some enzyme residue of strategic importance and observable by NMR, or by enabling an evaluation of the interaction coefficients, or by evidencing the changes intervening in the vicinity of the observed residue(s) when building step by step the catalytic complex, the use of Hill plots and their fitting to the relations derived here may contribute to a better understanding of catalytic mechanisms.

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References

Bovey FA (1969) NMR spectroscopy. Academic Press, New York, p 64

Dahlquist FW (1974) The quantitative interpretation of maximum in Scatchard plots. FEBS Lett 49: 267–268

Emsley JW, Feeney J, Sutcliffe LH (1965) High resolution NMR, vol 1. Pergamon Press, Oxford, p 485

Hill AV (1910) The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. J Physiol 40: IV–VIII

Markley JL (1975) Observation of histidine residues in proteins by means of NMR spectroscopy. Acc Chem Res 8: 70–80

Milner-White EJ, Watts DC (1971) Inhibition of adenosine 5′-triphosphate-creatine phosphotransferase by substrate-anion complexes. Evidence for the transition state organization of the catalytic site. Biochem J 122: 727–740

Roustan C, Pradel LA, Kassab R, Fattoum A, Thoai NV (1970) Spectrophotometric investigations of the interaction of native and chemically modified ATP: guanidinophosphotransferases with their substrates. Biochim Biophys Acta 206: 369–379

Sachs DH, Schechter AN, Cohen JS (1971) Nuclear magnetic resonance titration curves of histidine ring protons. I. Influence of neighbouring charged groups. J Biol Chem 246: 6576–6580

Schwarz G (1976) Some general aspects regarding the interpretation of binding data by means of a Scatchard plot. Biophys Struct Mech 2: 1–12

Shrager RI, Cohen JS, Heller SR, Sachs DH, Schechter AN (1972) Mathematical models for interacting groups in NMR titration curves. Biochemistry 11: 541–547
Tipton KF, Dixon HBF (1979) Effects of pH on enzymes. In: Purich DL (ed) Methods in enzymology, vol 63. Academic Press, New York, pp 183–213
Wyman J Jr (1948) Linked functions in heme proteins. Adv Protein Chem 4: 436–443
Wyman J Jr (1967) Allosteric linkage. J Am Chem Soc 89: 2202–2218

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