Relations between Protonation Constants and Titration Curves in Polyprotic Acids: A Critical View

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Three thermodynamically meaningful pK_a values can be defined for polyprotic acids: macroscopic, microscopic, and quasisite pK_a values. In this paper, the relation between these pK_a values and their relation to titration curves is discussed. Often inflection points of total and individual titration curves or the pH value where the proton binding site is half protonated, so-called p$K_{1/2}$ values, are used to identify the pK_a values of polyprotic acids or of a proton binding site within the polyprotic acids. However, both are generally not identical with the pK_a values of a polyprotic acid. The different thermodynamic definitions of pK_a values are compared to commonly used ways of obtaining pK_a values from titration curves. The inflection points and p$K_{1/2}$ values are a first good guess for further fitting. However, only fitting titration curves to proper thermodynamic expressions lead to the respective pK_a values that are associated with the reaction free energy. A polyprotic acid with N titratable groups has 2^N microstates and thus $2^N - 1$ independent microscopic constants. However, only $N^2 - N + 1$ parameters can be extracted from the titration curves of all individual sites. Because $2^N - 1$ is greater than $N^2 - N + 1$ for $N > 3$, it follows that it is impossible to obtain all microscopic constants from the titration curves of all individual sites for polyprotic acids with more than three nonidentical proton binding sites. For $N \leq 3$, it is explained how to obtain the microscopic constants from the titration curves of all individual sites using the decoupled sites representation. The method is applied to determine the microscopic constants of DTPA, which has highly irregular titration curves. From the microscopic constants, the state populations are calculated and the reason for the unusually shaped titration curve is explained.

Introduction

Proton binding is a very common and simple chemical reactions. The protonation of a molecule controls its charge and thus greatly influences the physical and chemical properties of the molecule. Understanding protonation equilibria is therefore crucial for understanding the chemistry and the reactivity of molecules.

The proton binding equilibrium is usually characterized by a pK_a value, which is directly proportional to the standard free energy of the protonation reaction. It is a common practice to use either the inflection point of titration curve or the pH at which the protonation probability is 0.5 (the so-called p$K_{1/2}$ value) to obtain the pK_a value. However, neither the inflection point nor the p$K_{1/2}$ value are definitions of the pK_a value. Just the character of the titration curve of monoprotic acids leads to the property that the pK_a value, the inflection point, and the p$K_{1/2}$ value all coincide. In contrast to the titration of monoprotic acids, the titration of polyprotic acids is usually more complicated because of the interaction between the protons bound to the different binding sites and because of the binding statistics. The inflection points and the p$K_{1/2}$ values of the titration curve coincide with pK_a values of polyprotic acids only under special circumstances. The complications that occur in polyprotic acids and the binding of several ligands to the same molecule in general are reviewed in several papers and books.[1−7] Recently, we could show that every total titration curve of a polyprotic acids with N sites can be described as a sum N noninteracting, so-called quasisites. The titration curves of the individual sites are just a linear combination of the titration curves of these quasisites.[8] The formalism to describe this relationship is called decoupled sites representation (DSR).

For polyprotic acids, one can define three different equilibrium constants: macroscopic, microscopic, and quasisite constants. This paper will discuss these different equilibrium constants and clarify the relation between them. Furthermore it will be explained how and under which circumstances the equilibrium constants can be obtained from titration curves. The effect of the ionic strength on the different equilibrium constants is not considered explicitely. It is assumed that the ionic strength does not change during the titrations.

The paper has the following structure. First, the basic principles of the titration of monoprotic acids are summarized. Next, diprotic acids are discussed in detail, because many principles of polyprotic acids can be understood from diprotic acids already. For diprotic acids, it is also investigated when p$K_{1/2}$ values and inflection points of titration curves are good approximations for pK_a values. The description is then generalized to polyprotic acids. A simple way to obtain microscopic equilibrium constants of di- and triprotic acids from the DSR by using simple linear fits of titration curves of individual sites is described. From the DSR, it follows that it is impossible to determine all microscopic equilibrium constants from the titration curves of the individual sites for molecules with more than three nonidentical binding sites, because the titration curves of individual sites do not contain enough information. The DSR formalism is applied to obtain the microscopic constants for diethylenetriaminepentaacetate (DTPA) which possesses a highly irregular titration curve. The physical basis of these

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irregular titration curves can be understood from the microscopic equilibrium constants. In the end, the main conclusions of the paper are summarized.

Titration of a Monoprotic Acid

The protonation equilibrium of a monoprotic acid can be described by eq 1

$$\mathrm{HA} \overset{K_a}{\rightleftharpoons} \mathrm{A^-} + \mathrm{H^+}; \quad K_a = \frac{[\mathrm{A^-}][\mathrm{H^+}]}{[\mathrm{HA}]} \tag{1}$$

where K_a is the equilibrium constant and $[A^-]$, [HA], and $[H^+]$ represent the concentration of the deprotonated species, the protonated, and the protons, respectively. The pH of the solution and the pK_a of an acid are defined as the negative decimal logarithm of the proton concentration (pH $= -\log [H^+]$) and the K_a value (pK_a $= -\log K_a$), respectively. Using these definitions, one obtains the Henderson−Hasselbalch equation from eq 1. The protonation probability $\langle x \rangle$ of a protonatable group is given by eq 2 which is algebraically equivalent to the Henderson−Hasselbalch equation with $\lambda = 10^{-\mathrm{pH}}$:

$$\langle x \rangle = \frac{10^{\mathrm{p}K_a - \mathrm{pH}}}{1 + 10^{\mathrm{p}K_a - \mathrm{pH}}} = \frac{K_a^{-1}\lambda}{1 + K_a^{-1}\lambda} \tag{2}$$

This equation describes a standard sigmoidal titration curve that is commonly found in text books. The denominator of eq 2 corresponds to the partition function of the molecular system, which becomes zero when $K_a = -\lambda$. By rearranging eq 2, it is possible to calculate the pK_a value from the protonation probability. From eq 2, one can see that the protonation probability is 0.5, when the pH equals the pK_a value. Furthermore, the pK_a value also coincides with the inflection point of the titration curve, i.e., the pH at which the titration curve has the steepest slope. At the inflection point, the second derivative of eq 2 becomes zero, which is exactly the case when pH value equals the pK_a value (see the Supporting Information).

For a monoprotic acid, the inflection point of the titration curve and the p$K_{1/2}$ value all coincide with the thermodynamically defined pK_a value. The pK_a value is defined from the mass law in eq 1 and has a clear relation to the standard reaction free energy (standard conditions, pH=0) for protonating a group, which is given by $G_a^0 = -\beta^{-1}$ ln 10 pK_a, where β is the reciprocal of the product of the universal gas constant and the temperature. The energy for protonating a group at a certain pH is given by $G_a = G_a^0 - \mu_{H^+} = -\beta^{-1}$ ln 10 (pK_a − pH), where μ_{H^+} is the chemical potential of the protons in the solution.

Titration of a Diprotic Acid

Thermodynamics and Titration Curves. The behavior of a diprotic acid will be described in detail for illustrating and summarizing the concepts required for polyprotic acids. Given is a system of two interacting proton binding sites. Such a system can adopt four states that are described by a protonation state vector **x**, where the components x_i mark whether site i is protonated ($x_i = 1$) or deprotonated ($x_i = 0$). For a system of two sites, one has the following states: both sites deprotonated (00), only the first site protonated (10), only the second site protonated (01), and both sites protonated (11; see Figure 1a). One can assign microscopic equilibrium constants $K_{\mathbf{r}}^{\mathbf{p}}$ to all four equilibria in this reaction scheme, where the subscript **r** denotes the protonation state vector of the reactant state and the superscript **p** denotes the protonation state vector of the product state. The microscopic equilibrium constants in eq 3

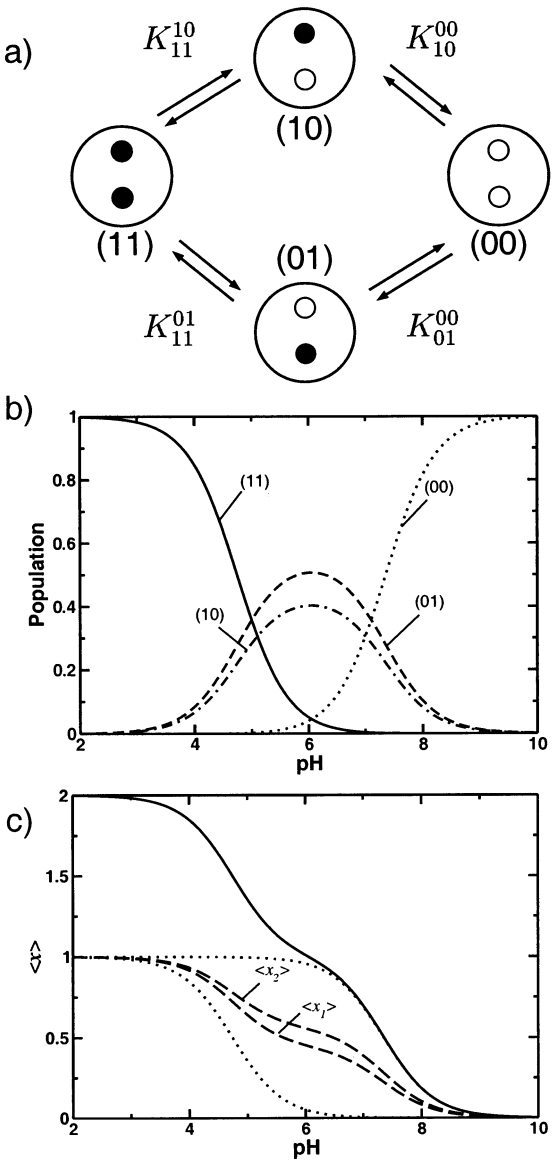

Figure 1. Titration of a diprotic acid having the microscopic values p$K_{00}^{10} = 7.0$ and p$K_{00}^{01} = 7.1$ and an interaction energy of $W = 2.0$ pK_a units. The quasisite pK'_j values of this system are 4.74 and 7.35, which are due to the strong interaction numerically identical with the macroscopic p$\bar{K}_k$ values. The a_{11} parameter of the system is 0.56. The other entries of the a_{ij} matrix can be obtained from eq 16. (a) Schematic representation of the equilibria between the different microspecies of the system. The protonation state vector **x** is given below the states. (b) Population of the microspecies. The protonation state vector **x** marks the population curves. (c) Total titration curve (solid line), titration curves of the individual sites (dashed lines) and quasisite titration curves (dotted lines).

are defined analogously to the equilibrium constants for monoprotic acids in eq 1:

$$K_{11}^{10} = \frac{[(10)][H]}{[(11)]}; \quad K_{11}^{01} = \frac{[(01)][H]}{[(11)]}$$
$$K_{10}^{00} = \frac{[(00)][H]}{[(10)]}; \quad K_{01}^{00} = \frac{[(00)][H]}{[(01)]} \tag{3}$$

The microscopic p$K_{\mathbf{r}}^{\mathbf{p}}$ values are defined as the negative decimal logarithm of the microscopic equilibrium constants $K_{\mathbf{r}}^{\mathbf{p}}$ (p$K_{\mathbf{r}}^{\mathbf{p}} = -\log K_{\mathbf{r}}^{\mathbf{p}}$), which are connected to the free energy difference of the relevant microstates $G_{\mathbf{p}}^0 - G_{\mathbf{r}}^0 = -\beta^{-1}$

ln 10 pK_r^p at standard conditions, i.e., pH = 0. Because the energy of the states must be independent from the way to reach the state, the following relation must hold $pK_{11}^{01} + pK_{01}^{00} = pK_{11}^{10} + pK_{10}^{00}$. Therefore, one can define an interaction energy W between the sites as $W = pK_{10}^{00} - pK_{11}^{01} = pK_{01}^{00} - pK_{11}^{10}$. With this definition, W should be positive; that is, binding of the first proton should disfavor the binding of the second because of electrostatic repulsion. Because protons should repel each other because of their charge, only repulsion (positive interaction energy W) is considered here. Negative interaction energies would correspond to an attraction and lead to cooperative binding which will be discussed in a separate publication (Onufriev & Ullmann, in preparation). It should be noted that the formalism described here also applies for cooperative binding. The three parameters pK_{01}^{00}, pK_{10}^{00}, and W characterize the energetics of the binding of two ligands to a molecule completely. The totally deprotonated state is set to be the reference state ($G_{00}^0 = 0$). The partition function Z in terms of microscopic pK_r^p values and in terms of the energy G_x^0 of the protonation states **x** is then given by eq 4

$$Z = 1 + 10^{pK_{01}^{00} - pH} + 10^{pK_{10}^{00} - pH} + 10^{pK_{01}^{00} + pK_{10}^{00} - W - 2pH}$$
$$= 1 + e^{-\beta(G_{01}^0 - \mu_{H+})} + e^{-\beta(G_{10}^0 - \mu_{H+})} + e^{-\beta(G_{11}^0 - 2\mu_{H+})} \quad (4)$$

where μ_{H^+} is the chemical potential of the protons which is given by β^{-1} ln 10 pH. One should note that $-\beta G_{11}^0/\ln 10 = pK_{10}^{00} + pK_{11}^{10} = pK_{01}^{00} + pK_{11}^{01} = pK_{01}^{00} + pK_{10}^{00} - W$. The population of each of the states can be calculated from eq 5

$$\langle(00)\rangle = \frac{1}{Z}; \quad \langle(10)\rangle = \frac{1}{Z} 10^{pK_{10}^{00} - pH}$$
$$\langle(01)\rangle = \frac{1}{Z} 10^{pK_{01}^{00} - pH}; \quad \langle(11)\rangle = \frac{1}{Z} 10^{pK_{01}^{00} + pK_{10}^{00} - W - 2pH} \quad (5)$$

The pH dependence of the population of the four microscopic states is depicted in Figure 1b.

The total average protonation $\langle X\rangle$ of a molecule is given by the sum of the probability of each state multiplied by the number of protons bound in this state: $\langle X\rangle = \langle(10)\rangle + \langle(01)\rangle + 2\langle(11)\rangle$. The total protonation is measured by techniques that look at the system as a whole such as for instance potentiometry or calorimetry. Such measurements are usually interpreted in terms of macroscopic pK_a values: $p\bar{K}_k$. Macroscopic constant describes the equilibrium between the kth and the $(k-1)$th macrostate of the molecule, not the equilibrium for individual sites or between microstates of the molecule. For a system with two binding sites, the macroscopic equilibrium constants $\bar{K}_1$ and $\bar{K}_2$ in terms of microstate population and in terms of microscopic equilibrium constants are given by eq 6

$$\bar{K}_1 = \frac{[(00)][H]}{[(10)] + [(10)]} = \frac{K_{10}^{00} K_{01}^{00}}{K_{10}^{00} + K_{01}^{00}}$$
$$\bar{K}_2 = \frac{([(10)] + [(10)])[H]}{[(11)]} = K_{11}^{10} + K_{11}^{01} \quad (6)$$

The macroscopic $p\bar{K}_k$ values are given as the negative decimal logarithm of the equilibrium constants ($p\bar{K}_k = -\log \bar{K}_k$). The partition function in eq 4 can be rewritten in terms of macroscopic equilibrium constants as given by eq 7

$$Z = 1 + 10^{p\bar{K}_1 - pH} + 10^{p\bar{K}_1 + p\bar{K}_2 - 2pH} \quad (7)$$

The values of $p\bar{K}_1$ and $p\bar{K}_2$ are obtained by fitting experimentally obtained macroscopic titration curves to eq 8

$$\langle X\rangle = \frac{10^{p\bar{K}_1 - pH} + 2 \times 10^{p\bar{K}_1 + p\bar{K}_2 - 2pH}}{1 + 10^{p\bar{K}_1 - pH} + 10^{p\bar{K}_1 + p\bar{K}_2 - 2pH}} \quad (8)$$

The titration curve of an individual site gives the probability that this site in the molecule is protonated at a given pH. They are therefore obtained by summing the probabilities of all states in which the site of interest is protonated, i.e., for a diprotic acid by $\langle x_1\rangle = \langle(10)\rangle + \langle(11)\rangle$ and $\langle x_2\rangle = \langle(01)\rangle + \langle(11)\rangle$ using eq 5. The titration curve of an individual site does not represent a microscopic titration curve but rather a sum of the population curves of all of the microstates that have this site protonated. Titration curves of individual sites of a molecule with two interacting sites are given in Figure 1c. Individual titration curves are measured by techniques that enable us to monitor the protonation of a particular site, such as for instance nuclear magnetic resonance (NMR).[9−16]

It is always possible to describe macroscopic titration curves in terms of the titration curves of noninteracting (decoupled) quasisites.[2,8,17,18] The quasisites are generally not identical with a particular proton binding site in the molecule. Usually many real sites of the molecule contribute to one quasisite. The pK_a values of these quasisites (pK'_j) are obtained as negative of the decimal logarithm of the negative of the roots of the partition function Z (eqs 4 and 7), where $\lambda = 10^{-pH}$ is the variable of the polynomial from which the roots are determined. The quasisite pK'_j values have a clear connection to the energetics of binding one proton to one quasisite: $\Delta G_j^{0'} = -\beta^{-1}$ ln 10 pK'_j. Analogously to a real protonation state vector **x**, it is possible to define a quasisite protonation state vector **y** where y_j is 1 or 0 depending whether quasisite j is protonated or not. The partition function written in terms of the pK'_j values of the decoupled quasisites and in terms of the energies $\mathcal{G}_y^0$ of the quasistates **y** is given by eq 9

$$Z = 1 + 10^{pK'_1 - pH} + 10^{pK'_2 - pH} + 10^{pK'_1 + pK'_2 - 2pH}$$
$$= 1 + e^{-\beta(\mathcal{G}_{01}^0 - \mu_{H+})} + e^{-\beta(\mathcal{G}_{10}^0 - \mu_{H+})} + e^{-\beta(\mathcal{G}_{11}^0 - 2\mu_{H+})} \quad (9)$$

where $\mathcal{G}_y^0 = y_1 \Delta G_1^{0'} + y_2 \Delta G_2^{0'}$. The partition functions in eqs 4, 7, and 9 are all fully equivalent and describe the same system. The partition function in eq 9 does, however, not contain an interaction term W in contrast to eq 4, because the quasisites do not interact. The DSR is still a microscopic representation; that is, one can still in principle differentiate between two states that have only a single proton bound.

We could demonstrate recently[8] that individual titration curves are just a linear combination of the two quasisite titration curves (eq 10)

$$\langle x_1\rangle = a_{11} \frac{10^{pK'_1 - pH}}{1 + 10^{pK'_1 - pH}} + a_{12} \frac{10^{pK'_2 - pH}}{1 + 10^{pK'_2 - pH}}$$
$$\langle x_2\rangle = a_{21} \frac{10^{pK'_1 - pH}}{1 + 10^{pK'_1 - pH}} + a_{22} \frac{10^{pK'_2 - pH}}{1 + 10^{pK'_2 - pH}} \quad (10)$$

where a_{ij} represent parameters that indicate how much the quasisite titration curve contributes to the individual titration curves. Because each real and also each quasisite can only bind one proton, one can introduce the following constraint $a_{11} +$

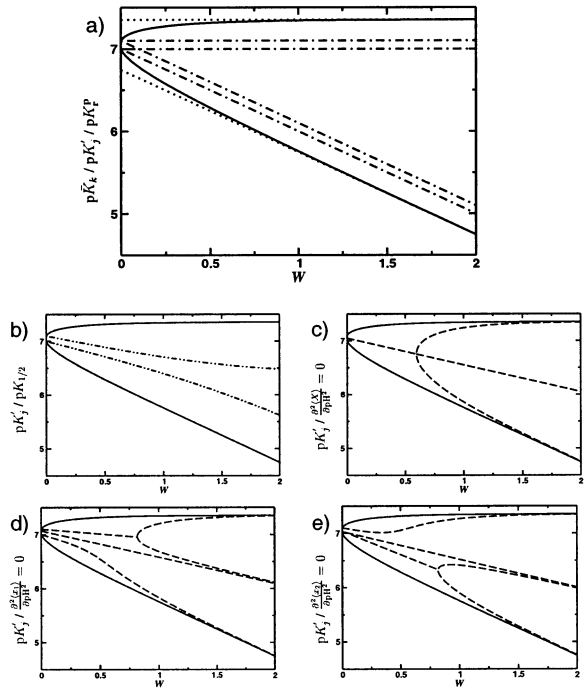

Figure 2. Comparison of thermodynamically-defined pK_a values with p$K_{1/2}$ values and inflection points of a diprotic acid in dependence on the interaction energy between the binding sites in pH units. The microscopic values pK_{10}^{00} and pK_{01}^{00} of this diprotic acid are set to 7.0 and 7.1, respectively. (a) Macroscopic p$\bar{K}_k$ values (dotted lines), microscopic pK_r^p (dot-dashed lines), and quasisite pK'_j values (solid lines) of a diprotic system in dependence on the interaction between the sites. (b) Comparison quasisite pK'_j values (solid lines) and p$K_{1/2}$ values (dot-dot-dashed lines). (c) Comparison quasisite pK'_j values (solid lines) and inflection points of the total titration curve (dashed lines). (d) comparison quasisite pK'_j values (solid lines) and inflection points of the titration curve of site 1 (dashed lines). (e) comparison quasisite pK'_j values (solid lines) and inflection points of the titration curve of site 2 (dashed lines). The bifurcation points in the panels c, d, and e mark the points where the inflection point get an imaginary part. Only the real part of the inflection points is shown.

$a_{12} = a_{21} + a_{22} = a_{11} + a_{21} = a_{12} + a_{22} = 1.0$. That means that only one parameter in the a_{ij} matrix of a diprotic acids is free. The a_{ij} matrix can be obtained from a microscopic model as outlined briefly below.

Relation between Titration Curves and pK_a Values for Diprotic Acids. Different ways of obtaining pK_a values from titration curves are used in practice. Here, the different, definitions and their applicability are compared. Three different thermodynamically meaningful pK_a values can be defined for polyprotic acids: microscopic pK_r^p values, macroscopic p$\bar{K}_k$ values, and quasisite pK'_j values. Figure 2 shows a comparison of these thermodynamically defined pK_a values with the inflection points and the p$K_{1/2}$ values of a diprotic acid. The microscopic values pK_{10}^{00} and pK_{01}^{00} of this diprotic acid are set to 7.0 and 7.1, respectively. The interaction energy between the two sites is used as a variable in Figure 2. The titration behavior of such a diprotic acid with an interaction energy of 2.0 pK_a units is depicted in Figure 1. The complications described here occur when the two binding sites titrate in the same pH range and interact. If the different proton binding sites titrate at very different pH, these complications do not occur even if there is a strong interaction. In the latter case, the site that titrates first (at low pH) sees the nontitrating site only in the protonated form. Then, when the second site titrates (at higher pH), the first site is already virtually completely deprotonated. When, however, both sites titrate in the same pH range, they interact with each other in the protonated and the deprotonated form during titration. This interaction causes irregular titration curves.

Figure 2a shows the macroscopic p$\bar{K}_k$ values (the two dotted lines), the microscopic pK_r^p values (the four dot−dashed lines), and the quasisite pK'_j values (the two solid lines) as a function of the interaction energy between the site. One can see that at large interaction energies, the macroscopic p$\bar{K}_k$ values and the quasisite pK'_j values coincide. When the interaction energy is zero, the microscopic pK_r^p and the quasisite pK'_j values are identical. All of the pK_a values in Figure 2a have a clearly defined thermodynamic meaning.

NMR measurements can deliver individual titration curves. In analogy to the titration of single protonatable groups, the pH at which the protonation is 0.5, so-called p$K_{1/2}$ value, is often used to indicate the titration behavior. In Figure 2b, the quasisite pK'_j values (the two solid line) and p$K_{1/2}$ value (the two dot-dot−dashed lines) are shown. It is obvious that the p$K_{1/2}$ values do not agree with quasisite pK'_j values. From comparison with Figure 2a, one sees that the p$K_{1/2}$ values do also not agree with the macroscopic p$\bar{K}_k$ values nor with the microscopic pK_r^p values, except when the sites do not interact. The p$K_{1/2}$ value cannot be used to describe the energetics of chemical reactions.

Also in analogy to the titration curves of monoprotic acids, the inflection points of the titration curves of polyprotic acids are sometimes used to estimate their pK_a values. The total titration curve of a molecule with two interacting has three inflection points (Figure 2c), and the titration curves of the individual sites have four inflection points (Figure 2d and e). Some of these inflection points may be complex numbers, depending on the interaction energy between the sites. In Figure 2c−e, only the real parts of the inflection points are shown as a function of the interaction between the sites. The inflection points are obtained by setting the second derivative of the total and the individual titration curves to zero (see the Supporting Information). Equations that relate the inflection points of two interacting groups with identical pK_{10}^{00} and pK_{01}^{00} values to the interaction energy W between them have been presented before.[22] However, also inflection points do not agree with quasisite pK'_j values, macroscopic p$\bar{K}_k$ values, or microscopic pK_r^p values of the system, except when the sites do not interact. For large interaction energies W, two of the inflection points, the quasisite pK'_j values and the macroscopic p$\bar{K}_k$ values, of the system coincide numerically. For the case of two identical strongly interacting sites, the equations for obtaining the quasisite pK'_j values take a simple mathematical form.[22] One can see that the inflection points of the total and the individual titration curves do not coincide if the two interacting groups are not identical, but approach each other for strong interaction energies W. The inflection points of neither the individual nor the total titration curves do represent any physically meaningful pK_a value. They are, however, good estimates for finding a first guess for fitting titration curves.

Using eq 2, it is possible after rearranging to calculate the pK_a value from the protonation probability. As one would expect, the pK_a value obtained this way does not depend on pH for monoprotic acids. For a polyprotic acid, however, one obtains then pH-dependent pK_a values. Figure 4 shows such a pH-dependent pK_a value (solid lines) for a diprotic acid in comparison to the macroscopic p$\bar{K}_k$ values and the quasisite pK'_j values (dashed lines, both are numerically identical for this system) and to the microscopic pK_r^p values (dotted lines).

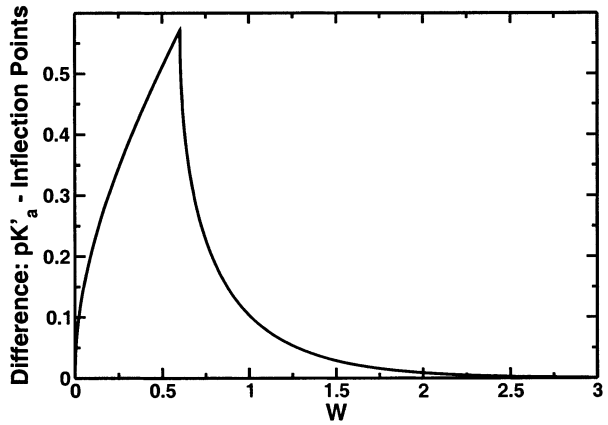

Figure 3. Differences between the inflection points of the titration curve and the quasisite pK'_j values of a system of two interacting identical sites having the microscopic binding constants $pK_{00}^{10} = pK_{00}^{01} = 7.0$ in dependence on the interaction W between the two sites.

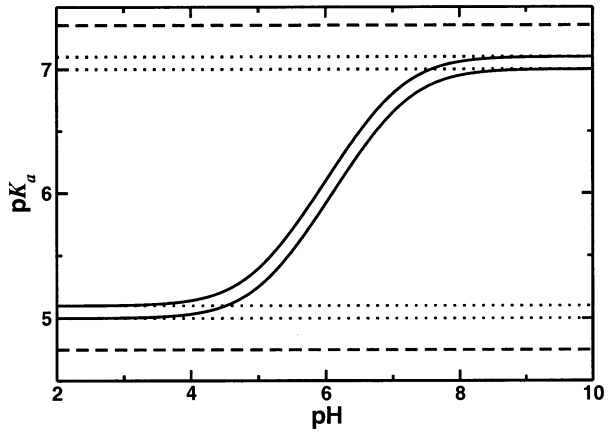

Figure 4. pH-dependent pK_a value of a diprotic acid having the microscopic values $pK_{00}^{10} = 7.0$ and $pK_{00}^{01} = 7.1$ and an interaction energy of $W = 2.0$ pK_a-units calculated from $pK_a = pH + \log(\langle x_i\rangle/(1 - \langle x_i\rangle))$ (solid lines) in comparison with the macroscopic $p\bar{K}_k$ values (dashed lines) and the microscopic $pK_{\mathbf{r}}^{\mathbf{p}}$ (dotted lines). The macroscopic $p\bar{K}_k$ values and the quasisite pK'_j values are numerically identical for this system.

When the two sites are nearly completely deprotonated or protonated, the pH-dependent pK_a value obtained from the protonation probability converges to the microscopic $pK_{\mathbf{r}}^{\mathbf{p}}$ values. In general, such pH-dependent pK_a values do not describe the energetics of the titration process properly and are only a mean field approximation.[19−21] The pH-dependent pK_a values describe the energy that is on average required to protonate the group of interest at a given pH.

To obtain the macroscopic $p\bar{K}_k$ values for experimental titration curves, eq 8 needs to be fitted to an experimentally determined total titration curve. Microscopic $pK_{\mathbf{r}}^{\mathbf{p}}$ values can be obtained by fitting the individual titration curves $\langle x_1\rangle = \langle(10)\rangle + \langle(11)\rangle$ and $\langle x_2\rangle = \langle(01)\rangle + \langle(11)\rangle$ using eq 5 or alternatively from the DSR as explained below. Quasisites pK'_j values can then be obtained from the roots of the partition function, which can be written either in terms of macroscopic $p\bar{K}_k$ values (eq 7) or microscopic $pK_{\mathbf{r}}^{\mathbf{p}}$ values (eq 4). The inflection points do not correspond to the macroscopic $p\bar{K}_k$ or the quasisite pK'_j values of the system. For small interaction energies W, the inflection point deviate considerably from thermodynamically meaningful pK_a values as can be seen in Figure 3. Only for large W, the inflection points and the macroscopic $p\bar{K}_k$ or the quasisite pK'_j values of the system coincide. The inflection points of the titration curves and the $pK_{1/2}$ values are, however, good first guesses for the $p\bar{K}_k$ and pK'_j values that need to be refined in a fit.

Titration of Polyprotic Acids

Thermodynamics and Titration Curves. Most of the statements made for diprotic acids hold for polyprotic acids with more than two sites. In particular, it is also true that neither the inflection points nor the so-called $pK_{1/2}$ values describe the energetics of proton titration, which is shown in more detail in the Supporting Information.

In analogy to eq 3, one can define microscopic equilibrium constants, and from these equilibrium constants, one can calculate the energy of each protonation state. Each protonation state can be represented by a N-dimensional protonation state vector $\mathbf{x}$ in which x_i is 1 and 0 depending on whether site i is protonated or not. The standard free energy $G_{\mathbf{x}}^0$ of the protonation state $\mathbf{x}$ is proportional to the sum of the microscopic $pK_{\mathbf{r}}^{\mathbf{p}}$ values, that describes the equilibrium between the state of interest and the reference state. The totally deprotonated state is set to be the reference state, and its energy is set to zero. The standard free energy G_{0111}^0 of the state (0111) of a tertaprotic acid is for instance given by $-\beta^{-1}\ln 10\ (pK_{0001}^{0000} + pK_{0011}^{0001} + pK_{0111}^{0011})$. Several different combinations of microscopic $pK_{\mathbf{r}}^{\mathbf{p}}$ values are possible to calculate the energy of the same state.

The partition function of a polyprotic acid in terms of microscopic constants is then given by eq 11

$$Z = \sum_{\mathbf{x}}^{2^N} e^{-\beta G_{\mathbf{x}}^0}\, e^{\beta n_{\mathbf{x}} \mu_{H+}} \tag{11}$$

where $n_{\mathbf{x}}$ is the number of protons bound to the molecule in the state $\mathbf{x}$ ($n_{\mathbf{x}} = \sum_{i=1}^{N} x_i$) and μ_{H^+} is the chemical potential of the protons which is given by $\beta^{-1}\ln 10\ pH$, where β is the reciprocal of the product of the universal gas constant and the temperature. The sum in eq 11 ranges over all 2^N possible protonation states. The partition function is a polynomial where $e^{\mu_{H+}}$ is the variable of the polynomial. Equation 11 is therefore also called binding polynomial.[18] The sum of the Boltzmann factors $e^{-\beta G_{\mathbf{x}}^0}$ of the states $\mathbf{x}$ each having $n_{\mathbf{x}}$ proton bound give the $n_{\mathbf{x}}$th coefficient of the polynomial. Several different states can have the same number $n_{\mathbf{x}}$ of protons bound, but the protons are distributed differently over the binding sites, for instance in the case of the states (1001) and (1010). The probability that a particular site i is protonated in the molecule, i.e., the titration curve of an individual site, is given by eq 12

$$\langle x_i\rangle = \frac{1}{Z}\sum_{\mathbf{x}}^{2^N} x_i\, e^{-\beta G_{\mathbf{x}}^0}\, e^{\beta n_{\mathbf{x}} \mu_{H+}} \tag{12}$$

The macroscopic $p\bar{K}_k$ values describe the i th protonation equilibrium of the molecule as a whole. The kth macroscopic $p\bar{K}_k$ value is given by eq 13

$$p\bar{K}_k = -\log\left(\frac{\sum_{\mathbf{x}}^{2^N}\delta(k-1)\, e^{-\beta G_{\mathbf{x}}^0}}{\sum_{\mathbf{x}}^{2N}\delta(k)\, e^{-\beta G0_{\mathbf{x}}^0}}\right) \tag{13}$$

where $\delta(k)$ is 1.0 if the state $\mathbf{x}$ has k protons bound ($n_{\mathbf{x}} = k$) and otherwise 0.0. A macroscopic $\mathrm{p}\bar{K}_k$ value is therefore a thermodynamic average over all equilibria connected to the release of the kth proton. The sum of the first up to the kth macroscopic $\mathrm{p}\bar{K}_k$ value give the kth polynomial coefficient of the partition function in eq 11. The macroscopic $\mathrm{p}\bar{K}_k$ values can consequently also be used to write the partition function and total titration of polyprotic acids are commonly interpreted in terms of macroscopic $\mathrm{p}K_a$ values.

The titration of polyprotic acids can be described in terms of decoupled quasisites.[2,3,8,17,18] The $\mathrm{p}K'_j$ values of these quasisites are obtained as negative of the decimal logarithm of the negative of the roots of the partition function Z in eq 11. The titration curves of every individual site $\langle x_i \rangle$ in a polyprotic acid with N proton binding sites can be expressed as a linear combination of the N titration curves of N independent quasisites $\langle y_j \rangle$[8] as shown in eq 14

$$\begin{pmatrix} \langle x_1 \rangle \\ \langle x_2 \rangle \\ \vdots \\ \langle x_N \rangle \end{pmatrix} = \begin{pmatrix} a_{11} & a_{12} & \cdots & a_{1N} \\ a_{21} & a_{22} & \cdots & a_{2N} \\ \vdots & \vdots & \cdots & \vdots \\ a_{N1} & a_{N2} & \cdots & a_{NN} \end{pmatrix} \begin{pmatrix} \langle y_1 \rangle \\ \langle y_2 \rangle \\ \vdots \\ \langle y_N \rangle \end{pmatrix} \tag{14}$$

or simpler in eq 15

$$\langle x_i \rangle = \sum_{j=1}^{N} a_{ij} \langle y_j \rangle \tag{15}$$

Each of the quasisite titration curves $\langle y_j \rangle$ has a sigmoidal shape as given by eq 2. Because each real and also each quasisite can only bind one proton, the sum over all row and also the sum over all columns in the a_{ij} matrix must be one[8] (eq 16)

$$\sum_{i=1}^{N} a_{ij} = \sum_{j=1}^{N} a_{ij} = 1 \tag{16}$$

The coefficient matrix is obtained from the solution of N sets of N-dimensional systems of linear equations, which is explained in more detail in Onufriev et al.[8] Here, only a short version of the derivation is given. Analogously to real protonation states, one can define quasi-state vectors $\mathbf{y}$ where y_j is 1 or 0 depending if quasisite j is protonated or not. The standard free energy of a microquasistate $\mathscr{G}^0_y$ in terms quasisite $\mathrm{p}K'_j$ values is simply given by eq 17

$$\mathscr{G}^0_y = -\beta^{-1} \ln 10 \sum_{j=1}^{N} y_j \, \mathrm{p}K'_j \tag{17}$$

The average protonation of a quasisite is given by eq 18

$$\langle y_j \rangle = \frac{1}{Z} \sum_{\mathbf{y}}^{2^N} y_j^k \, \mathrm{e}^{-\beta \mathscr{G}^0_y} \, \mathrm{e}^{\beta n_y \mu_{H+}} \tag{18}$$

The solutions of the systems of equations in eq 19 leads to the a_{ij} matrix. There are a total of N systems such as eq 19, one for every $i = 1, \ldots, N$

$$\begin{pmatrix} \sum_x^{2^N} x_i \, \delta(1) \, \mathrm{e}^{-\beta G^0_{\mathbf{x}}} \\ \sum_x^{2^N} x_i \, \delta(2) \, \mathrm{e}^{-\beta G^0_{\mathbf{x}}} \\ \vdots \\ \sum_x^{2^N} x_i \, \delta(N) \, \mathrm{e}^{-\beta G^0_{\mathbf{x}}} \end{pmatrix} = \begin{pmatrix} \sum_y^{2^N} y_1 \, \delta(1) \, \mathrm{e}^{-\beta G^0_{\mathbf{y}}} & \sum_y^{2^N} y_2 \, \delta(1) \, \mathrm{e}^{-\beta G^0_{\mathbf{y}}} & \cdots & \sum_y^{2^N} y_N \, \delta(1) \, \mathrm{e}^{-\beta G^0_{\mathbf{y}}} \\ \sum_y^{2^N} y_1 \, \delta(2) \, \mathrm{e}^{-\beta G^0_{\mathbf{y}}} & \sum_y^{2^N} y_2 \, \delta(2) \, \mathrm{e}^{-\beta G^0_{\mathbf{y}}} & \cdots & \sum_y^{2^N} y_N \, \delta(2) \, \mathrm{e}^{-\beta G^0_{\mathbf{y}}} \\ \vdots & \vdots & \cdots & \vdots \\ \sum_y^{2^N} y_1 \, \delta(N) \, \mathrm{e}^{-\beta G^0_{\mathbf{y}}} & \sum_y^{2^N} y_2 \, \delta(N) \, \mathrm{e}^{-\beta G^0_{\mathbf{y}}} & \cdots & \sum_y^{2^N} y_N \, \delta(N) \, \mathrm{e}^{-\beta G^0_{\mathbf{y}}} \end{pmatrix} \times \begin{pmatrix} a_{i1} \\ a_{i2} \\ \vdots \\ a_{iN} \end{pmatrix} \tag{19}$$

The factors $x_i \, \delta(n)$ and $y_j \, \delta(n)$ filter out the states that have n protons bound and that are protonated at site i or at quasisite j, respectively.

The description of individual titration curves of a molecule in terms of quasisites is as general as the statistical mechanical description provided in eq 12. The a_{ij} matrix indicates how much the jth quasisite $\mathrm{p}K'_j$ value contributes to the total protonation of the ith real site. Negative values in the a_{ij} matrix mark a partial transfer of protons from the real site i to other sites when the jth quasisite gets protonated.

Binding Sites Combinatorics in Polyprotic Acids. A molecule with N proton binding sites can exist in $N + 1$ macrostates (no proton bound, one proton bound, etc. up to N protons bound) and 2^N microstates. The number p_i of microstates that have i protons bound is given by eq 20

$$p_i = \binom{N}{i} = \frac{N!}{(N-i)!\, i!} \tag{20}$$

The number of possible equilibrium constants between the states having i and states having $(i + 1)$ proton bound to N sites is given by the number of states having i protons bound times the number of empty sites, which is $N - i$, because the protons can only bind to the empty sites. The total number of possible microscopic equilibrium constants is thus $N2^{N-1}$. However, only $2^N - 1$ of these equilibrium constants are independent, because one can calculate the microscopic equilibrium constants from the difference between the free energies of the 2^N microstates. The energy of one state, the reference state, can be arbitrarily set to zero. The number of macroscopic binding constants is N.

The DSR[8] gives a complete description of the individual titration curves. Each of the titration curves of individual sites in a molecule with N binding sites can be described as a linear combination of up to N quasisites titration curves. The coefficients a_{ij} of the linear combination form a $N \times N$ matrix. The a_{ij} matrix contains $(N - 1)^2$ independent parameters. The number of quasisite $\mathrm{p}K_a$ values is N. Therefore, all titration curves of individual sites in a molecule can be described by $N + (N - 1)^2 = N^2 - N + 1$ parameters. Vice versa that means that, even if all individual titration curves are measured, the curves contain only the information for $N^2 - N + 1$ parameters.

Table 1 lists the number of microconstants, the number of independent microconstants, and the number of DSR parameters required to describe all individual titration curves for molecules with up to six proton binding sites. One can see that for molecules with up to three proton binding sites the number of

TABLE 1: Listing of the Number of Equilibrium Constants and DSR Parameters for Molecules with up to Six Proton Binding Sites

binding sites	DSR-parameters	independent microscopic constants	microscopic constants
N	$N^2 - N + 1$	$2^N - 1$	$2^{N-1}N$
1	1	1	1
2	3	3	4
3	7	7	12
4	13	15	32
5	21	31	80
6	31	63	192

parameters that can describe individual titration curves and the number of independent microconstants are identical. That means one can determine the microscopic binding constants from the titration curves of the individual sites for di- and triprotic acids. For molecules with more than three proton binding sites, it is not possible anymore to determine the microscopic binding constants from the individual titration curves. For polyprotic acids with $N > 3$, the number of microscopic equilibrium constants exceeds the number of parameters that are required to describe individual titration curves. For tetraprotic acid, it is possible to extract 13 parameters from all individual titration curves, but 15 independent microscopic pK_r^p values are required to fully characterize the system. For more sites, this ratio becomes even more unfavorable. Only for systems with symmetry or with special assumption, one can determine microscopic equilibrium constants from titration curves of individual sites of polyprotic acids with more than three nonidentical sites. A fit of titration curves of individual sites to microscopic equilibrium constants using eq 12 without special assumptions is an over-fitting for polyprotic acids with $N > 3$. Even if the titration curves of all individual proton binding sites are measured with infinite precision, they can never contain the information of all microscopic constants if the molecule has more than three proton binding sites and no symmetry.

Obtaining Microscopic Equilibrium Constants from Individual Titration Curves of Di- and Triprotic Acids. With techniques such as NMR, it is possible to follow the titration of a particular site in a polyprotic acids and thus to measure individual titration curves.[9−16] As outlined above, it is possible to derive all microscopic constants from the individual titration curves for di- and triprotic acids. For higher polyprotic acids, it is only possible to obtain all microscopic constants if it is possible to reduce the number of independent microscopic constants to $N^2 - N + 1$, i.e., to the number of DSR parameters that are required to describe all individual titration curves. The number of parameters can reduce if the molecule is symmetric or if some microscopic constants can be assumed to be identical. If this is possible, a scheme similar to the one outlined below will allow to determine microscopic constants.

Using the DSR, one can derive from eq 19 that the microscopic constants for a diprotic acid are given by eq 21

$$pK_{10}^{00} = \log(a_{11}10^{pK'_1} + a_{12}10^{pK'_2})$$

$$pK_{01}^{00} = \log(a_{21}10^{pK'_1} + a_{22}10^{pK'_2})$$

$$pK_{11}^{10} = \log\left(\frac{a_{11}10^{pK'_1+pK'_2} + a_{12}10^{pK'_1+pK'_2}}{a_{11}10^{pK'_1} + a_{12}10^{pK'_2}}\right)$$

$$pK_{11}^{01} = \log\left(\frac{a_{21}10^{pK'_1+pK'_2} + a_{22}10^{pK'_1+pK'_2}}{a_{21}10^{pK'_1} + a_{22}10^{pK'_2}}\right) \qquad (21)$$

For a triprotic acid, similar equations can be derived, which are however a bit more complicated. The equations for microscopic constants of di- and triprotic acids are derived in detail in the Supporting Information.

The advantage of using the DSR for obtaining microscopic pK_r^p values from experimentally measured titration curves is that data obtained from different types of experiments can be combined, and the fitting procedure is simple. Potentiometric measurements deliver macroscopic constants and thus the partition function in terms of these macroscopic constants. The quasisite constants can be calculated from the roots of the partition function. There are N macroscopic constants and also N quasisite constants for a molecule with N proton binding sites. The quasisite constants can be used in eq 14 to obtain the a_{ij} matrix from titration curves of individual sites from a *linear* fit. Additional constraints can be used in the fit, because the sum over all rows and over all columns in the a_{ij} matrix must be 1.0. For a diprotic acid, there is only one free parameter available to fit the individual titration curves of both sites if the macroscopic constants are already determined before. In the case of triprotic acids, there are only four free parameters to fit the individual titration curves of all three sites. Also for higher polyprotic acids, one can apply the same procedure to obtain the quasisite constants and the a_{ij} matrix. However, only with special assumptions, it is possible to determine microscopic constants from these parameters, because the number of microscopic constants exceeds the number of parameters that are required to describe individual titration curves.

Example: DTPA. Diethylenetriaminepentaacetate (DTPA) is one of the simplest molecules that show a complex titration behavior.[10,23] This molecule, shown in Figure 5a, has three amine nitrogens, and each of them can bind a proton. The individual titration curves have been measured by NMR.[10,23] The individual titration curves of the two terminal nitrogens and the central nitrogens are given in Figure 5, parts b and c, respectively. Because the molecule is symmetric, the two terminal nitrogens cannot be distinguished and their individual titration curves are identical. In a previous paper,[8] we have shown that the DSR model describes the individual titration curves very well. The DSR parameters that describe the individual titration curves are given in Table 2. The protonation states vector marks the protonation of the left, the central, and the right nitrogen. Using the DSR parameters in Table 2, we obtain the microscopic pK_r^p values which are listed in the same table. The equations to obtain the microscopic pK_r^p values are obtained from eq 19 and are derived in detail in the Supporting Information.

From the microscopic pK_r^p values one can obtain the population probabilities of the individual states in dependence on pH. Figure 5b shows the population of all states that contribute to the titration of the terminal nitrogens. Because the two terminal nitrogens are identical, only the curves for the left nitrogen is shown here. Figure 5c shows the population of all states that contribute to the titration of the central nitrogens. Adding up the curves of the population of the relevant states leads to the individual titration curve of these sites, which are also shown in Figure 5.

The titration curve of the central nitrogen is unusual because of its nonmonotonic behavior. The protonation probability increases with increasing pH (i.e., decreasing proton concentration). In the a_{ij} matrix, this unusual behavior is reflected by the negative matrix element a_{22} (see Table 2). This negative value

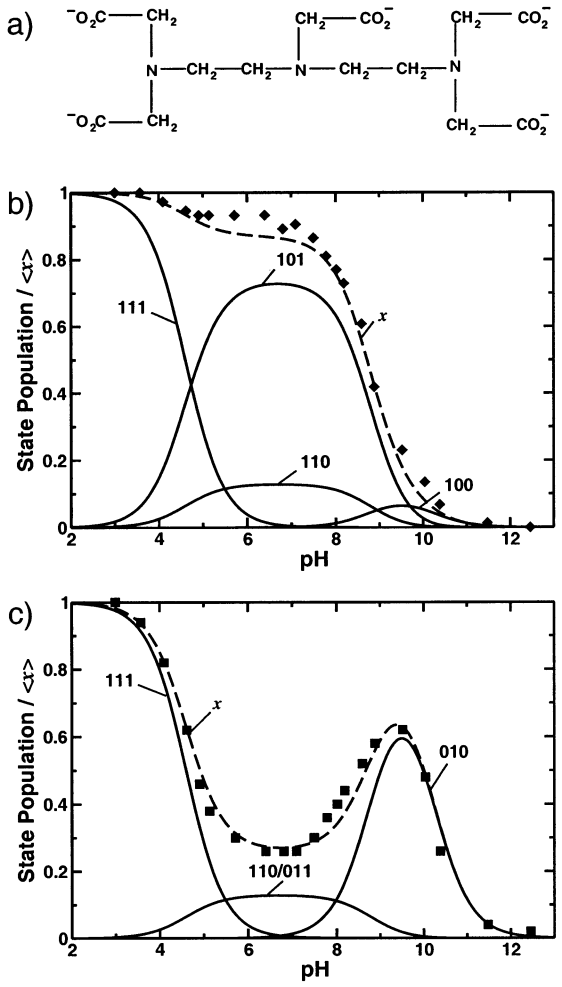

Figure 5. Titration of diethylenetriaminepentaacetate (DTPA). (a) Schematic representation of the molecule. b) Titration curve of the terminal left nitrogen measured by NMR[10] (diamonds) and fitted to the DSR (dashed line). The population curves of all microstates that contribute to the titration of this site are shown and marked in the diagram. The titration curves of the left and the right terminal nitrogen are identical. (c) Titration curve of the central nitrogen measured by NMR[10] (squares) and fitted to the DSR (dashed line). The population curves of all microstates that contribute to the titration of this site are shown and marked in the diagram.

indicates that a proton is transferred from proton binding site 2 (central nitrogen) to other sites in the molecules when a proton binds to the second quasisite.

The population of the microstates give a physical rational for the unusual, irregular titration behavior of the central nitrogen of DTPA. At high pH (low proton concentration), the protons bind preferable to the central nitrogen, because the binding affinity of a proton to the middle nitrogen is higher (see Table 2). Binding the second proton to one of the terminal nitrogens while the middle nitrogen stays protonated is unfavorable, because these two proton binding sites are in close proximity and the positively charged protons repel each other. It is therefore more favorable to deprotonate the central nitrogen when the second proton proton binds and rather protonate both terminal nitrogens. The two protons are at a greater distance from each other and thus repel each other less, when the two terminal nitrogens are protonated compared to protonating one terminal and the central. The energetics of proton binding is reflected in the microstate population in Figure 5 and in the microscopic constants in Table 2.

TABLE 2: Macroscopic $p\bar{K}_k$ Values, Quasisite pK'_j and Transformation a_{ij} Matrix (DSR parameters), and Microscopic pK_r^p Values of DTPA[a]

3. H^+		2. H^+		1. H^+	
Macroscopic $p\bar{K}_k$ Values					
$p\bar{K}_3$	4.6	$p\bar{K}_2$	8.8	$p\bar{K}_1$	10.2
Quasisite pK'_j Values					
pK'_3	4.6	pK'_2	8.8	pK'_1	10.2
a_{ij} Matrix					
a_{13}	0.13	a_{12}	0.81	a_{11}	0.06
a_{23}	0.74	a_{22}	−0.62	a_{21}	0.88
a_{33}	0.13	a_{32}	0.81	a_{31}	0.06
Microscopic pK_r^p Values					
pK_{111}^{110}	4.3	pK_{110}^{010}	8.9	pK_{100}^{000}	9.2
pK_{111}^{101}	5.3	pK_{110}^{100}	8.0	pK_{010}^{000}	10.1
pK_{111}^{011}	4.3	pK_{011}^{010}	8.9	pK_{001}^{000}	9.2
		pK_{011}^{001}	8.0		
		pK_{101}^{100}	9.7		
		pK_{101}^{001}	9.7		

[a] The macroscopic constants and the quasisite constants are numerically identical for this molecule because of the strong electrostatic interaction.

Conclusions

The titration of polyprotic acids is considerably more complicated than that of monoprotic acids. The titration curves of individual sites in polyprotic acids can be irregular and do not need to show a sigmoidal shape. Such irregular titration curves can be obtained experimentally from NMR and infrared spectroscopy or theoretically from titration curve calculations using the Poisson−Boltzmann equation and thermodynamic averaging. For irregular titration curves, the $pK_{1/2}$ value, i.e., pH at which the protonation probability is 0.5, or the inflection points of the titration curves of individual sites or of the total titration curve cannot be identified with the pK_a value. Only real thermodynamically defined pK_a values can be used to describe the energetics of protonation reactions. Inflection points and $pK_{1/2}$ values are, however, good estimates for pK_a values and thus good starting values for fitting pK_a values to titration curves. Total titration curves can always be described as a sum of the titration curves of noninteracting groups and therefore do not give any information about the complexity of the individual titration curves. More generally, titration curves measured by every method that investigates the pH dependence of the energetics of the system as a whole[24−28] can be described in terms of independent sites.

In polyprotic acids, one can differentiate between macroscopic, microscopic, and quasisite equilibrium constants. The microscopic constants describe the binding equilibria between the different binding states of a molecule, which consider all interactions between the binding sites. They can be related to macroscopic binding constants which describe how protons bind to the molecule as a whole. From these macroscopic constants, it is possible to obtain quasisite constants, i.e., microscopic constants, which assume no interaction between the binding sites. These quasisite constants cannot be related to particular sites of the molecule. However, the titration curves of the quasisites are related by a linear transformation to the titration curves of the real sites. Quasisite constants are microscopic constants but describe a decoupled system, i.e., a system without interaction between the binding sites. The relation between the different constants and the titration curves is graphically summarized in Figure 6.

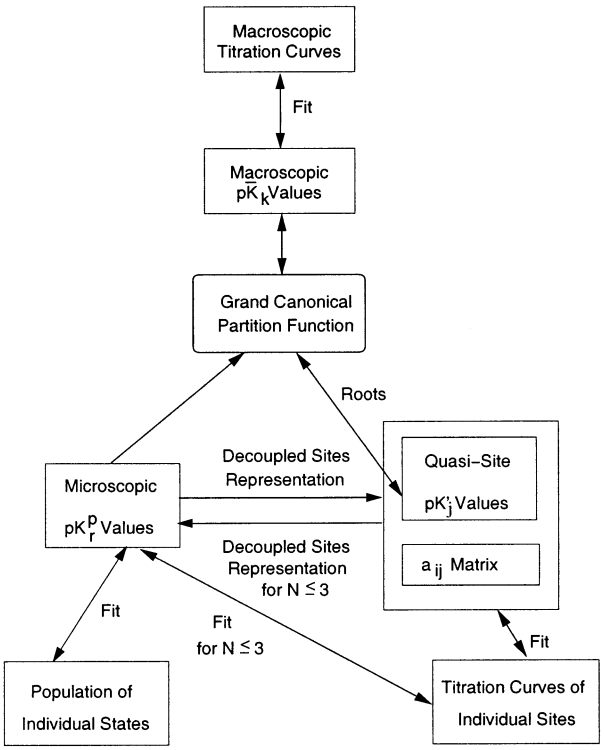

Figure 6. Graphical summary of the relation between macroscopic $p\bar{K}_k$ values, microscopic pK_r^p values, and quassi-site pK'_j values and of these constants to experimentally determined titration curves. Some of the arrows point only in one direction which indicates that these steps are only possible in one direction.

A polyprotic acid with N proton binding sites has $N2^{N-1}$ microscopic equilibrium constants, of which only $2^N - 1$ are independent. However, only $N^2 - N + 1$ parameters can be extracted from the titration curves of all individual sites. Therefore, the titration curves of individual sites cannot reflect all microscopic equilibria for polyprotic acids with more than three nonidentical proton binding sites. Hence, it is impossible to determine all microscopic equilibrium constants for molecules with more than three nonidentical sites from the titration curves of the individual sites, even if they are measured with infinite precision. For di- and triprotic acids, it is possible to get microscopic constants from the individual titration curves. For polyprotic acids with $N > 3$, additional information or special assumptions are required.

The whole formalism can be used to describe the binding of multiple ligands in general and is not restricted to protons. For example, binding of magnesium ions to nucleic acids or of zinc or calcium to proteins are another potential application. The decoupled sites representation is also applicable to the binding of regulatory proteins to DNA or the binding of ligands to oligomeric enzymes.

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Supporting Information Available: Two texts with figures and equations. Inflection Points of Titration Curves. How to Get Microscopic Constants from the Decoupled Sites Representation. This material is available free of charge via the Internet at http://pubs.acs.org.

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