Eur Biophys J (1999) 28: 533–551

REVIEW

G. Matthias Ullmann · Ernst-Walter Knapp

Electrostatic models for computing protonation and redox equilibria in proteins

Received: 11 March 1999 / Revised version: 24 May 1999 / Accepted: 26 May 1999

Introduction

Electrostatic interactions are the most relevant for understanding biochemical systems. Acid-base and redox reactions create or destroy unit charges in biomolecules and can thus be fundamental for their function. Besides association reactions and chemical modifications such as phosphorylations, they are the reason for changes in protein properties. Protonation or deprotonation of titratable groups can cause changes in binding affinities, enzymatic activities, and structural properties. Very often, protonations or deprotonations are the key events in enzymatic reactions. The reduction or oxidation of redox-active groups has a similar importance. In particular, the reduction of disulfide bonds can cause unfolding or functionally important conformational transitions. Consequently, the function of most proteins depends crucially on the pH and on the redox potential of the solution.

Acidic denaturation of proteins in the stomach is a prerequisite for protein degradation during digestion. Beside this rather unspecific effect, the environment can tune the physiological properties of proteins in a specific manner. Different values of pH or redox potential in different organs, tissues, cells, or cell compartments steer protein function. Physiological redox and pH buffers such as glutathione and phosphates control these environmental parameters in living systems strictly. A few examples emphasize the physiological importance of pH and redox potential. The pH gradient in mitochondria or chloroplasts drives ATP synthesis. This pH gradient is in both systems generated by several proton transfer steps that couple to a sequence of redox reactions. In hemoglobin, pH influences O_2 binding and thus regulates O_2 release during blood circulation. This behavior is also known as the Bohr effect. Pepsinogen cleaves itself in an acidic environment to the highly active peptidase pepsin. Membrane fusion during influenza virus infection involves large pH-induced structural changes of the protein hemagglutinin.

Because of their outstanding significance, electrostatic interactions in proteins have been investigated intensively in the last decades (for review see Warshel and Russel 1984; Harvey 1989; Sharp and Honig 1990; Bashford 1991; Warshel and Åqvist 1991; Moult 1992; Madura et al. 1994; Sharp 1994; Gilson 1995; Honig and Nicholls 1995; Beroza and Case 1998; Warshel and Papazyan 1998). Several different approaches are used to describe the electrostatics of proteins. The most detailed descriptions can be made by quantum-chemical approaches (Szabo and Ostlund 1989; Ziegler 1991; Lowe 1993; Naray-Szabó and Ferenczy 1995; Baerends and Gritsenko 1997). Such computationally expensive methods, however, only work for relatively small molecules and peptides. A cruder approximation must be chosen for larger systems such as solvated proteins. Molecular mechanics force fields are widely used for that purpose (Karplus and Petsko 1990; van Gunsteren and Berendsen 1990; Brooks and Case 1993; Kollman 1993). In these force fields, electrostatic interactions are modeled by (screened) Coulomb potentials. Most often, the solvent is considered explicitly in these approaches, i.e., at a microscopic level. In these simulations, fractions of unit charges – so-called atomic partial charges – are assigned to each atom. The atomic partial charges are adjusted to fit the electrostatic potential obtained from a quantum-chemical calculation of small molecules with equivalent chemical groups (Breneman and Wiberg 1990). Such atomic partial charges are also used in continuum electrostatic approaches that rely on the so-

G.M. Ullmann[1] · E.-W. Knapp (✉)
Institut für Kristallographie,
Freie Universität Berlin, Takustrasse 6,
D-14195 Berlin, Germany
e-mail: knapp@chemie.fu-berlin.de

Present address:
[1] Department of Molecular Biology,
The Scripps Research Institute,
10550 N. Torrey Pines Road,
TPC-15, La Jolla, CA 92037, USA

lution of the Poisson-Boltzmann equation (PBE; Harvey 1989; Sharp and Honig 1990; Bashford 1991; Madura et al. 1994; Sharp 1994; Gilson 1995; Honig and Nicholls 1995) or on the generalized Born model (Still et al. 1990; Schaefer and Karplus 1996; Schaefer et al. 1998). In these models the protein is represented as a continuum with a low dielectric constant and individual atomic partial charges, while the solvent is represented as a continuum with a high dielectric constant and without individual charges. Mobile salt ions of the solution are described by an ionic strength. This model provides a macroscopic description of electrostatic interactions of the solvent with a dissolved protein whereas the protein is still represented in atomic detail. Warshel and co-workers (Warshel and Russel 1984; Warshel and Åqvist 1991) developed an alternative approach that represents the solvent by space-fixed dipoles – so-called Langevin dipoles – while the protein is modeled by a conventional force field. Consequently, this description corresponds to a semi-microscopic representation of the protein-solvent system.

In this paper, we review the use of macroscopic solvent models to determine protonation and redox equilibria in proteins. Specifically, we will focus on methods that are able to calculate the protonation equilibria of proteins that have many titratable groups. Some of the methods reviewed here can account for conformational changes going along with a change of the protonation pattern. Furthermore, we discuss problems and possible future developments in this field as well as some of the recent applications.

Fundamental description of acid-base and redox reactions

The protonation equilibrium of a single titratable group can be described by Eq. (1), where K_a is the equilibrium constant:

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

The pH of the solution and the pK_a of an acid are defined as the negative decadic logarithm of the hydrogen ion concentration ($pH = -\log[H^+]$) and the K_a value ($pK_a = -\log K_a$), respectively. Using these definitions one obtains the Henderson-Hasselbalch equation [Eq. (2)] from Eq. (1):

$$pH = pK_a + \log\frac{[A^-]}{[HA]} \tag{2}$$

The relation between pK_a and the standard reaction free energy G_a° is given by $G_a^\circ = -RT pK_a \ln 10$, where R is the gas constant and T is the temperature. The probability $\langle x\rangle$ that the acid HA is protonated is given by $\langle x\rangle = [HA]/([HA]+[A^-])$. With this definition and Eqs. (1) and (2) one obtains

$$\langle x\rangle = \frac{\exp(-\ln 10(pH - pK_a))}{1+\exp(-\ln 10(pH - pK_a))} \tag{3}$$

From Eq. (3) one can read that $RT\ln 10(pH - pK_a)$ equals the free energy required to protonate a titratable group at a given pH and temperature.

The description of redox equilibria is in principle very similar to that of acid-base equilibria. The equilibrium between the redox couple A_{ox}/A_{red}^- is defined in Eq. (4):

$$A_{ox} + e^- \overset{K_{ET}}{\rightleftharpoons} A_{red}^-; \quad K_{ET} = \frac{[A_{red}^-]}{[A_{ox}][e^-]} \tag{4}$$

where K_{ET} is the equilibrium constant. Analogously to the pH and the pK_a in derivation of the Henderson-Hasselbalch equation [Eq. (2)], one defines the redox potential of the solution or the electromotive force and the standard redox potential of the redox couple A_{ox}/A_{red}^- as $E = -RT/F\ln[e^-]$ and $E^\circ = RT/F\ln K_{ET}$, respectively. Here, however, the natural logarithm rather than the decadic logarithm is used. With the factor RT/F, where F is the Faraday constant in A s, one obtains E and E° in volts. The relation between E° and the standard reaction free energy G_{et}° is given by $G_{et}^\circ = FE^\circ$. With these definitions and Eq. (4) one obtains the Nernst equation [Eq. (5)], which is analogous to the Henderson-Hasselbalch equation [Eq. (2)]:

$$E = E^\circ + \frac{RT}{F}\ln\frac{[A_{ox}]}{[A_{red}^-]} \tag{5}$$

The probability $\langle y\rangle$ that a redox-active group is in its oxidized state is defined as $\langle y\rangle = [A_{ox}]/([A_{red}^-]+[A_{ox}])$. In analogy to Eq. (3), the relation between the probability $\langle y\rangle$, the redox potential of the solution E, and the standard redox potential E° is given by Eq. (6):

$$\langle y\rangle = \frac{\exp\left(\frac{F}{RT}(E-E^\circ)\right)}{1+\exp\left(\frac{F}{RT}(E-E^\circ)\right)} \tag{6}$$

Consequently, the free energy required to oxidize a redox-active group at a given redox potential of the solution E equals $-F(E-E^\circ)$.

Computation of the electrostatic potential by the Poisson-Boltzmann equation

The electrostatic potential $\phi(\mathbf{r},\rho)$ at position $\mathbf{r}$, arising from the charge distribution $\rho(\mathbf{r})$ in an inhomogeneous dielectric medium is defined by the PBE [Eq. (7)]:

$$\nabla[\varepsilon(\mathbf{r})\nabla\phi(\mathbf{r})] = -4\pi\left(\rho(\mathbf{r}) + \sum_{i=1}^{K} c_i^{bulk} Z_i e_0 \exp\left(\frac{-Z_i e_0\phi(\mathbf{r})}{RT}\right)\right) \tag{7}$$

where ∇ is gradient operator with respect to the spatial coordinates, $\varepsilon(\mathbf{r})$ is the spatially varying dielectric constant, c_i^{bulk} is the concentration of ions i in the bulk and

Z_i is their charge. For small electrostatic potentials ($e_0\phi(\mathbf{r})/RT < 1$), the PBE can be linearized by expanding the exponential up to the linear term [Eq. (8)]:

$$\sum_{i=1}^{K} c_i^{\text{bulk}} Z_i e_0 \exp\left(\frac{-Z_i e_0 \phi(\mathbf{r})}{RT}\right) \cong \sum_{i=1}^{K} c_i^{\text{bulk}} Z_i e_0 - \sum_{i=1}^{K} c_i^{\text{bulk}} Z_i^2 e_0^2 \frac{\phi(\mathbf{r})}{RT} \quad (8)$$

The first term in Eq. (8) vanishes because of electroneutrality of the solution. It is useful to define the ionic strength I [Eq. (9)] and the inverse Debye length κ [Eq. (10)]:

$$I = \frac{1}{2}\sum_{i=1}^{K} c_i^{\text{bulk}} Z_i^2 \quad (9)$$

$$\kappa^2(\mathbf{r}) = \frac{8\pi e_0^2 I(\mathbf{r})}{RT} \quad (10)$$

With the definitions in Eqs. (9) and (10), and with Eqs. (7) and (8), one obtains the linearized Poisson-Boltzmann equation [LBPE; Eq. (11)]:

$$\nabla[\varepsilon(\mathbf{r})\nabla\phi(\mathbf{r})] = -4\pi\rho(\mathbf{r}) + \kappa^2\phi(\mathbf{r}) \quad (11)$$

In the LBPE, the electrostatic potential is additive as long as the dielectric boundaries of the molecular system remain unchanged. This means that if $\rho = \rho_1 + \rho_2$ then $\phi(\mathbf{r}, \rho) = \phi(\mathbf{r}, \rho_1) + \phi(\mathbf{r}, \rho_2)$ is valid.

Analytical solutions of the LPBE exist only for simple geometries (Kirkwood 1934; Daune 1997). For complex geometries, solutions can be obtained by numerical methods. Most often, the PBE is solved by finite difference methods (see Honig and Nicholls 1995 for a review), but also other numerical methods to treat partial differential equations such as boundary element methods (Sklenar et al. 1990; Zauhar and Varnek 1996) or multigrid-based methods (Holst et al. 1994; Holst and Saied 1995) can be used to solve the PBE or its linearized form. Most often, finite difference approximations are used for pK_a calculations, although boundary element methods are sometimes also applied (Ripoll et al. 1996; Juffer et al. 1997).

The value of the dielectric constant in proteins

Which value of the dielectric constant is appropriate for proteins is widely and controversially discussed (Harvey 1989; Gilson 1995; Warshel and Papazyan 1998). Antosiewicz et al. (1994) obtained best agreement with experiments by using a dielectric constant of 20 for the protein, rather than the commonly used dielectric constant of 4. These results were obtained with the so-called "full-charge model", i.e., a unit charge is placed on one atom of a charged titratable group. In a more recent study, however, they could show that the use of a "detailed charge model", i.e., when the charge is distributed over all atoms of the titratable group, yields agreement with experiments when a dielectric constant of 4 was used (Antosiewicz et al. 1996a). Demchuck and Wade (1996) analyzed the dependence of calculated pK_a values on the dielectric constant carefully. They made the interesting observation that a large dielectric constant led to better agreement with experiments for residues that are on the surface of the proteins, while for residues that are buried in the protein better results were obtained with a small dielectric constant. This observation is in good agreement with the calculation of the dielectric constants in proteins (Simonson et al. 1992; Simonson and Perahia 1995a, b; Simonson and Brooks 1996). The dielectric constant at the more flexible surface of proteins turned out to be about 10–20, while it was about 2–4 in the interior of the protein. The inclusion of individual water molecules in computations of pK_a values in proteins does not improve the agreement with experimental results (Gibas and Subramaniam 1996). This may be due to the unknown orientation of water molecules in crystal structures (Ullmann et al. 1996; Kannt et al. 1998; Rabenstein et al. 1998a, b). Furthermore, water molecules may reorient when a nearby titratable group changes its protonation. This would, however, require the inclusion of molecular flexibility in titration calculations, which is not done so far.

Quantum-chemical calculations of pK_a values and standard redox potentials $E°$

In order to calculate the energy required to protonate a titratable group inside a protein, it is necessary to know the energy required to protonate an isolated titratable group of the same kind, a so-called model compound. The same holds for a redox-active group. This energy can be measured experimentally, but in some cases the experimental values for suitable model compounds are not available or it is even impossible to synthesize proper model compounds. This is for instance the case for some metal centers in proteins which cannot form in solution. In these cases, quantum-chemical calculations are required to determine the appropriate energies. A large variety of methods exist to estimate acidities (Lim et al. 1991; Chen et al. 1994; Potter et al. 1994; Mousca et al. 1995; Chandra and Goursot 1996; Li et al. 1996; Merrill and Kass 1996; Kallies and Mitzner 1997; Richardson et al. 1997; Topol et al. 1997; Vilaiño et al. 1997). Noodleman, Bashford, Case, and co-workers developed a quantum-chemical method that considers the environment by a dielectric continuum model (Lim et al. 1991; Chen et al. 1994; Mousca et al. 1995; Li et al. 1996; Richardson et al. 1997; Li et al. 1998).

Quantum-chemical corrections to electrostatic energies

Characterizing energetics of proton or electron transfer in molecular systems requires the calculation of energy changes ΔE between different states of molecular groups

involved in these processes. We will henceforth call the molecular groups participating actively in the considered redox or protonation process the "relevant system" and the remaining part of the molecular system the "molecular environment". If conformational changes going along with the changes of the redox or protonation state are ignored, the main contribution to the energy change of these processes is of electrostatic nature because the charge state of the relevant system changes. The electrostatic energy ΔE_{elec} is calculated from a molecular model with atomic partial charges using an inhomogeneous dielectric to represent the protein-water-system. However, there are also corrections which appear only at the level of ab initio quantum-chemical computations $\Delta E_{\mathrm{qc-corr}}$ and cannot be accounted for by simple electrostatic energy terms, i.e., $\Delta E = \Delta E_{\mathrm{elec}} + \Delta E_{\mathrm{qc-corr}}$. An exact quantum-chemical treatment of the complete molecular system would consider all energy contributions but is practically impossible.

There are two possible approximation procedures to determine the quantum-chemical correction $\Delta E_{\mathrm{qc-corr}}$. One uses a quantum-chemical method to estimate the energy difference $\Delta E_{\mathrm{model}}$ for a small molecular model system which normally would involve the relevant system and possibly some molecular groups in its vicinity under vacuum conditions. The other possibility is to take the energy difference $\Delta E_{\mathrm{model}}$ from experimental data of a molecular model system which contains the relevant system or a molecular system close enough to the relevant system considered. Comparing these energies with the values obtained from an evaluation of the corresponding electrostatic energies $\Delta E_{\mathrm{model-elec}}$ of the model system, the quantum-chemical correction of the model system can be obtained from the relation $\Delta E_{\mathrm{qc-corr}} = \Delta E_{\mathrm{model}} - \Delta E_{\mathrm{model-elec}}$ and used as the quantum-chemical correction for the energies of the molecular system considered. The underlying assumption is that the quantum-chemical corrections to the energy do not change if the relevant system is embedded in different molecular environments.

Quantum-chemical corrections to the electrostatic energy are due to the breaking or formation of covalent bonds (cov) and self-polarization effects within the relevant system (sp) and polarization effects induced by interactions with the molecular environment (ip). Thus we have $\Delta E_{\mathrm{qc-corr}} = \Delta E_{\mathrm{cov}} + \Delta E_{\mathrm{sp}} + \Delta E_{\mathrm{ip}}$. Energy contributions from self-polarization are accounted for properly by using quantum-chemical computations for the relevant system only. Screening electrostatic interactions by using a dielectric constant larger than unity can reduce the strength of polarization effects but not replace them. Electronic polarization (ep) and nuclear polarization (np) can be discriminated, such that $\Delta E_{\mathrm{ip}} = \Delta E_{\mathrm{np}} + \Delta E_{\mathrm{ep}}$. Normally, electrostatic energies of molecular systems are calculated only by considering one molecular conformation for each state of the relevant system. Under these circumstances, nuclear polarization effects are ignored. There are two types of interactions involving electronic polarization effects. One is the interaction of charges from one molecule polarizing the electronic wave function of a neighbor molecule, which we call charge-polarization (cp); the other is the attractive interaction of two molecules by mutual polarization (pp) of their electronic wave functions, $\Delta E_{\mathrm{ep}} = \Delta E_{\mathrm{cp}} + \Delta E_{\mathrm{pp}}$.

For acid-base and redox reactions the relevant system changes its total charge by one unit, giving rise to considerable changes of its molecular charge distribution. Hence, besides electronic self-polarization effects within the relevant system, a dominant part of the energy differences from charge-polarization is due to interactions of charges from the relevant system with the polarized electronic wave functions from the molecular environment (rcp). The second contribution to the energy difference is due to a change of the polarizability of the relevant system interacting with charges from the molecular environment (ecp), $\Delta E_{\mathrm{cp}} = \Delta E_{\mathrm{rcp}} + \Delta E_{\mathrm{ecp}}$. While the changes in the charge distribution of the relevant system occurring during a transition of the redox or protonation state are expected to be large, changes in the polarization of the relevant system will generally be smaller. Hence, the corresponding energy contribution ΔE_{ecp} will be smaller than ΔE_{rcp}.

For a molecular environment consisting of small polar molecules like water which undergo fast reorientational motions, the contribution of electronic polarization of the environment is approximately replaced by nuclear polarization if the atomic partial charges are enlarged correspondingly. In energy functions for proteins, the strength of H-bonds involving also polarization effects is adjusted by enlarging the atomic partial charges of the atoms participating in the H-bond. In general this procedure would work for all immobilized molecular groups in biological macromolecules, though it is not well justified. A better way to calculate the energy contribution ΔE_{rcp} is to use suitable values for the atomic partial charges of the different states of the relevant system and the corresponding values for the polarizabilities of the molecular environment. Consideration of these energy contributions would lead to a major improvement for the computed values of electrostatic energies.

While the attractive $1/r^6$ terms of conventional Lennard-Jones interactions account for the interaction due to mutually induced electronic polarization of all atomic groups of a molecular system in the electronic ground state, no suitable parameters are available for different charge and electronic states involving the relevant system. A molecular mechanics force field being able to account for changes in electronic polarization going along with a change of the redox or protonation state of the relevant system would require a major effort to calculate the polarizabilities for the different charge states of the molecular relevant system by quantum-chemical methods. The resulting energy corrections will be smaller than corrections from charge-polarization effects (cp). Hence, as long as one neglects electronic polarization effects of the type ecp, there is no need to consider also adjusted Lennard-Jones interactions.

Nuclear polarization contributions to energy differences between the different charge states of the relevant system can be calculated using molecular mechanics energy functions with flexible molecular models and suitable values of the atomic partial charges accounting for the electrostatic potential in the vicinity of the relevant system. Since the changes in the molecular conformations should be consistent with the molecular environment, not only electrostatic energy terms but also bonded and conventional Lennard-Jones interactions should be considered in this case. The quality of the results may depend on details of the procedure. If the conformation of the molecular environment is adjusted to a change of the charge state of the relevant system by energy minimization only, the resulting energy values may agree better with experimental data if Lennard-Jones interactions are not considered (Ullmann et al. 1996).

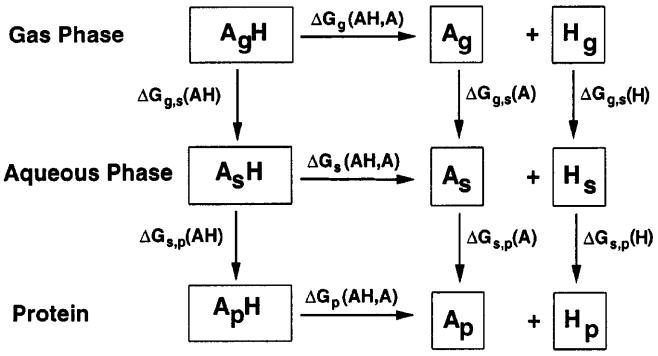

Fig. 1 Thermodynamic cycle to calculate the protonation energy from gas phase properties. In the first step the molecule is transferred from the gas phase (g) to a solution (s) and in a second step the molecule is transferred from the solution into the protein (p). The pK_a is in principle given by $pK_a = \frac{1}{\ln 10 RT}\Delta G_s(AH,A) = \frac{1}{\ln 10 RT}[\Delta G_g(AH,A) + \Delta G_{g,s}(A) - \Delta G_{g,s}(AH) + \Delta G_{g,s}(H)]$. The calculation scheme given in Eqs. (13)–(15) is however required to cancel artificial energy contributions that are caused by the finite difference method used to solve the Poisson-Boltzmann equation

Titration of a protein in a single conformation

The calculation of protein titration curves involves two steps. In the first step, the intrinsic pK_a values and the interaction energies between titratable groups are computed. In the second step, these energy terms are used to calculate the protonation probabilities of the titratable groups in a protein. The partition in two independent steps relies on the additivity of the electrostatic potentials obtained from the LPBE. These steps are described in the two subsequent sections.

Computation of the protonation state energies

The pK_a value of a titratable group within a protein can be considerably shifted compared to the pK_a value of the same titratable group in aqueous solution. The shift is caused by interactions between the charges of the titratable group and other charges in the protein and also by changes in the dielectric environment of the titratable group when the group is transferred from aqueous solution into the protein. If the protonation of only a single titratable group in the protein depends on pH, the pK_a shift is related to the difference between protonation energy of this group in the protein and the protonation energy of a proper model compound. The shifts of the pK_a values can be obtained via the thermodynamic cycles shown in Fig. 1. The pK_a values of model compounds in aqueous solution are usually determined experimentally (see Tables 1 and 2) or corresponding energies are calculated quantum chemically. The thermodynamic cycle in Fig. 1 illustrates the calculation of the shift of the pK_a value due to the transition of the titratable group from the gas phase or the aqueous solution to the protein environment. The thermodynamic basis and the underlying assumptions that allow the discrimination of a system into a quantum mechanical, a classical mechanical, and a continuum electrostatic region are given in detail by Gilson and co-workers (Gilson et al. 1997; Luo et al. 1998).

Quite often the protonation of more than one titratable group in a protein depends on pH. Thus the interaction between these titratable groups is also pH dependent. Owing to the interactions between these groups, titration curves of amino acids in proteins can deviate considerably from sigmoidal Henderson-Hasselbalch titration curves as given by Eq. (19). Because of these interactions, it is sometimes impossible to assign a unique pK_a value to a specific titratable group. Therefore, the pH value at which the protonation probability of the titratable group is 0.5 is often used instead to describe the titration behavior. This so-called $pK_{1/2}$ value does not directly relate to an energy difference and is thus not appropriate to discuss the energetics of catalytic mechanisms. Equation (12) defines the pK_a value on the basis of the protonation probability $\langle x \rangle$:

$$pK_a = pH + \frac{1}{\ln 10} \ln \frac{\langle x \rangle}{1 - \langle x \rangle} \tag{12}$$

This definition is more appropriate to discuss energetic issues at a given pH value. According to this definition, the pK_a value depends explicitly and implicitly on pH, because the protonation probability $\langle x \rangle$ depends on pH. Both pH dependencies cancel for standard Henderson-Hasselbalch-type titration curves, yielding a pH-independent pK_a value.

Often electrostatic interactions are the predominant contributions that cause the difference between the protonation energies of a titratable group in a protein and in aqueous solution. Then, the PBE or its linearized form provides a reasonable approximation of this energy difference. The additivity of the solutions of LPBE makes it possible to separate the protonation energy of a titratable group in a protein into several independent contributions. Therefore, the method described here is exact in the limit

Table 1 pK_a values of model compounds for biologically relevant titratable groups

Titratable group	Model compound pK_a	Ref.
α-Carboxyl group	3.8	Nozaki and Tanford (1967)
α-Amino group	7.5	Nozaki and Tanford (1967)
Aspartate	4.0	Nozaki and Tanford (1967)
Glutamate	4.4	Nozaki and Tanford (1967)
Cysteine pK_1	9.5	Nozaki and Tanford (1967)
Tyrosine pK_1	9.6	Nozaki and Tanford (1967)
Arginine	12.0	Nozaki and Tanford (1967)
Lysine	10.4	Nozaki and Tanford (1967)
Tryptophane	16.8	Yagil (1967)
Histidine $pK_{a,1}$ ($N_{\delta 1}$)[a]	7.0	Tanokura (1983)
Histidine $pK_{a,1}$ ($N_{\varepsilon 2}$)[b]	6.6	Tanokura (1983)
Histidine $pK_{a,2}$	14.0	Kaim and Schwederski (1995)
Heme Propinate	4.8	Moore and Pettigrew (1990)
Phosphate $pK_{a,1}$	1.96	Holleman and Wiberg (1964)
Phosphate $pK_{a,2}$	6.92	Holleman and Wiberg (1964)
Phosphate $pK_{a,3}$	11.72	Holleman and Wiberg (1964)
Sulfate $pK_{a,1}$	1.8	Weast (1986)
Sulfate $pK_{a,2}$	6.9	Weast (1986)
Water $pK_{a,1}$	−1.7	Weast (1986)
Water $pK_{a,2}$	15.7	Weast (1986)
Glycerol-2-phosphate $pK_{a,1}$[c]	1.3	Tanford (1962)
Glycerol-2-phosphate $pK_{a,2}$[c]	6.6	Tanford (1962)
Glucose-1-phosphate $pK_{a,2}$[c]	6.5	Tanford (1962)

[a] $N_{\delta 1}$ is methylated
[b] $N_{\varepsilon 2}$ is methylated
[c] As models for phosphorylated sugars

Table 2 Redox potentials $E^{\circ\prime}$ of model compounds for biologically relevant groups at pH = 7 (biological standard)

Redox couple	$E^{\circ\prime}$ (mV)	Ref.
Heme model (Met, His)	−70	Wilson (1983)
Heme model (His, His)	−220	Wilson (1983)
Tyrosine$\cdot^{+}$/tyrosine	+930	Cramer and Knaff (1991)
$O_2/O_2\cdot^{-}$	−330	Cramer and Knaff (1991)
Pheophytin	−600	Cramer and Knaff (1991)
FMN ox/sq (pH 7.0)	−238	Rao et al. (1993)
FMN sq/red (pH 7.0)	−172	Rao et al. (1993)

of the LPBE. The non-linear PBE (NPBE) has sometimes been used to calculate pK_a values with the framework reviewed in this article (Oberoi and Allewell 1993; Ripoll et al. 1996). However, the applicability of the NPBE, for which the contributions to the electrostatic potential are non-additive, has never been studied systematically.

One contribution to the protonation state energy arises from the protonation of an isolated model compound (see Fig. 2) of the titratable group μ. This energy contribution is related to the pK_a of this model compound in aqueous solution, $pK_{a,\mu}^{\text{model}}$. Transferring the titratable group μ from aqueous solution into a protein, in which all other titratable groups are in their uncharged protonation form, causes an energy shift. This energy shift consists of two contributions. The first energy contribution $\Delta\Delta G_\mu^{\text{Born}}$ is a Born energy term [Eq. (13)], which arises from the interaction of the partial charges $Q_{i,\mu}$ of the titratable group μ with its reaction field:

$$\Delta\Delta G_\mu^{\text{Born}} = \frac{1}{2}\sum_{i=1}^{N_\mu} Q_{i,\mu}^{\text{h}}[\phi_{\text{p}}(\mathbf{r}_i; Q_\mu^{\text{h}}) - \phi_{\text{m}}(\mathbf{r}_i; Q_\mu^{\text{h}})] - \frac{1}{2}\sum_{i=1}^{N_\mu} Q_{i,\mu}^{\text{d}}[\phi_{\text{p}}(\mathbf{r}_i; Q_\mu^{\text{d}}) - \phi_{\text{m}}(\mathbf{r}_i; Q_\mu^{\text{d}})] \qquad (13)$$

The second energy contribution $\Delta\Delta G_\mu^{\text{back}}$ arises from the interaction of the charges $Q_{i,\mu}$ of the titratable group μ with background charges of non-titrating groups and with the charges of the uncharged form of all other titratable groups in the protein [Eq. (14)]:

$$\Delta\Delta G_\mu^{\text{back}} = \sum_{i=1}^{N_{\text{p}}} q_i[\phi_{\text{p}}(\mathbf{r}_i; Q_\mu^{\text{h}}) - \phi_{\text{p}}(\mathbf{r}_i; Q_\mu^{\text{d}})] - \sum_{i=1}^{N_{\text{m}}} q_i[\phi_{\text{m}}(\mathbf{r}_i; Q_\mu^{\text{h}}) - \phi_{\text{m}}(\mathbf{r}_i; Q_\mu^{\text{d}})] \qquad (14)$$

The summations in Eq. (13) run over the N_μ atoms of group μ that have different charges in the protonated (h) ($Q_{i,\mu}^{\text{h}}$) and in the deprotonated (d) ($Q_{i,\mu}^{\text{d}}$) form. The first summation in Eq. (14) runs over the N_{p} charges of the protein that belong to atoms in non-titratable groups or to atoms of titratable groups ($\nu \neq \mu$) in their uncharged protonation form. The second summation in Eq. (14) runs over the N_{m} charges of atoms of the model compound that do not have different charges in the different protonation forms. The upper case letters Q refer to charges of titratable groups; lower case letters q denote charges of the reference protonation state, i.e., charges

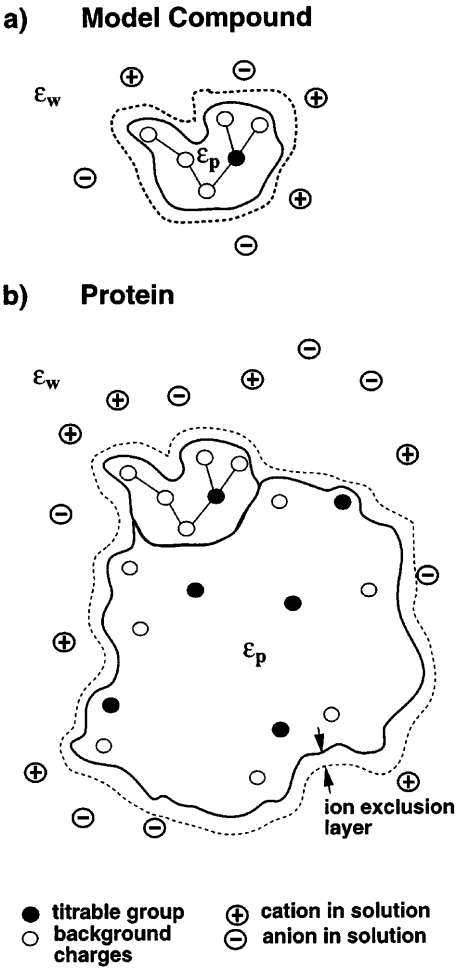

Fig. 2 Titratable groups and model compound in an ionic solution. The model compound needs to have the same conformation as the amino acid in the protein in order to maintain the cancellation of the artificial grid energy using the thermodynamic cycle technique

of non-titratable groups and charges of the titratable groups in their uncharged protonation form. The terms $\phi_m(\mathbf{r}_i, Q_\mu^h), \phi_m(\mathbf{r}_i, Q_\mu^d), \phi_p(\mathbf{r}_i, Q_\mu^h)$, and $\phi_p(\mathbf{r}_i, Q_\mu^d)$ denote the values of the numerical solutions of the LPBE at the position $\mathbf{r}$ of the atom i. The numerical solution of the LPBE was obtained using the shape of either the protein (subscript p) or the model compound (subscript m) as dielectric boundary and assigning the charges of the titratable group μ in either the protonated (Q_μ^h) or the deprotonated (Q_μ^d) form to the respective atoms. These two energy contributions and the pK_a value of the model compound are combined to give the so-called intrinsic pK_a value, p$K_{a,\mu}^{intr}$ [Eq. (15)]:

$$pK_{a,\mu}^{intr} = pK_{a,\mu}^{model} - \frac{1}{RT \ln 10}(\Delta\Delta G_\mu^{Born} + \Delta\Delta G_\mu^{back}) \quad (15)$$

The intrinsic pK_a value is the pK_a value that this particular titratable group would have if all other titratable groups are in their reference protonation form, i.e., the neutral protonation form. The interaction $W_{\mu\nu}$ between two titratable groups μ and ν in their charged form is defined in Eq. (16):

$$W_{\mu\nu} = \sum_{i=1}^{N_\mu} [Q_{\mu,i}^h - Q_{\mu,i}^d][\phi_p(\mathbf{r}_i, Q_\nu^h) - \phi_p(\mathbf{r}_i, Q_\nu^d)] \quad (16)$$

The energy of a protonation state n of the protein, which is characterized by the protonation state vector $\vec{x}_n = (x_1^n, x_2^n, \ldots, x_N^n)$, is given by Eq. (17):

$$G^n = \sum_{\mu=1}^{N} \left((x_\mu^n - x_\mu^0) RT \ln 10 (\mathrm{pH} - \mathrm{p}K_{a,\mu}^{intr}) \right) + \frac{1}{2} \sum_{\mu=1}^{N} \sum_{\nu=1}^{N} (W_{\mu\nu}(x_\mu^n + z_\mu^o)(x_\nu^n + z_\nu^o)) \quad (17)$$

where the x_μ^n are 1 or 0 depending whether group μ is protonated or not, and z_μ^o is the unitless formal charge of the deprotonated form of group μ, i.e., -1 for acids and 0 for bases. The sums run over all N titratable groups. Here and in all subsequent equations it is assumed that $W_{\mu\mu} = 0$. The additional x_μ^0-term of the intrinsic pK_a^--values refers to the reference protonation state (0). It redefines the zero point of the energy such that G^n vanishes for the reference protonation state. This leads to significant simplifications of the expressions needed if different conformations are considered, see below. Equivalent expressions are used in basically all approaches that solve the LPBE for obtaining titration curves (Bashford and Karplus 1990, 1991; Beroza et al. 1991; Yang et al. 1993; Antosiewicz et al. 1996a; Beroza and Fredkin 1996).

If the molecular system contains beside the N titratable groups also K redox-active groups, Eq. (17) needs an additional term that accounts for the redox potential E of the solution, as shown in Eq. (18) (Ullmann 1998):

$$G^n = \sum_{\mu=1}^{N} ((x_\mu^n - x_\mu^0) RT \ln 10 (\mathrm{pH} - \mathrm{p}K_{a,\mu}^{intr})) - \sum_{\eta=1}^{K} (x_\eta^n F(E - E_\eta^{\circ,intr})) + \frac{1}{2} \sum_{\nu=1}^{N+K} \sum_{\mu=1}^{N+K} (W_{\nu\mu}(x_\mu^n + z_\mu^o)(x_\nu^n + z_\nu^o)) \quad (18)$$

Here, $E_\eta^{\circ,intr}$ is the intrinsic standard redox potential of the redox-active group η; for redox-active groups, x_η^n is either 1 or 0 depending whether group η is oxidized or not; z_η^o is the formal charge of the reduced form of group η. The sum in the last term runs over all titratable and redox-active groups. With this approach, also the coupling between redox and protonation reactions can be investigated.

In the framework of this description, the problem of calculating the energy of each of the 2^N protonation states of a protein with N titratable groups is scaled down. N intrinsic pK_a values and $(N \times (N-1))/2$ interaction energies $W_{\nu\mu}$ are computed, which are used for the evaluation of the energies of the 2^N protonation states. Instead of solving the LPBE for the protein 2^N

times numerically, i.e., once for each protonation state, only $2 \times N$ numerical solutions of the LPBE for the protein are needed. In addition, $2 \times N$ numerical solutions of the LPBE for the model compounds are required no matter if the protonation state energy is computed as a sum over intrinsic pK_a values and interaction energies or directly from the LPBE for each protonation state. The calculation of the energy of a particular protonation state n of the protein is given by Eq. (17). This energy can in principle be used in Eq. (19) to obtain protonation probabilities as the thermodynamic average of the protonation of group μ, but a complete evaluation of the sum constituting the thermodynamic average is too time consuming for proteins with many titratable groups. Therefore, approximation methods have been developed to reduce the computational burden (Tanford and Roxby 1972; Bashford and Karplus 1991; Gilson 1993; Yang et al. 1993) which are described in the next section.

Calculation of the protonation probability of titratable groups in a protein

For the sake of simplicity, we no longer discriminate between titratable and redox-active groups and denote them both as titratable. If both, titratable and redox-active groups, are involved, Eq. (18) will have to be used to calculate state energies. All approximation methods presented here are, however, easily adjustable to compute also oxidation probabilities. Several strategies to calculate protonation probabilities of proteins or other biomolecules exist in the literature. Here, we describe only those that are most commonly used.

Direct evaluation of the statistical average

Assuming that each titratable group has only two possible protonation forms, the total number of protonation states of a protein with N titratable groups is 2^N. The protonation state of the protein can be described by a N-component vector $\vec{x} = \left(x_1^n, x_2^n, \ldots, x_N^n\right)$. The components x_μ of that vector adopt either the value 1 or 0 depending on whether group μ is protonated or deprotonated. The protonation probability $\langle x_\mu \rangle$ of the group μ is given by a thermodynamic average over all possible protonation states of the protein given by Eq. (19):

$$\langle x_\mu \rangle = \frac{\sum_{i=1}^{2^N} x_\mu^i \exp(-G^i/RT)}{\sum_{i=1}^{2^N} \exp(-G^i/RT)} \tag{19}$$

where x_μ^i is one or zero depending whether group μ is protonated in the protonation state i or not. G^i is the free energy of the protonation state i. From the probability $\langle x_\mu \rangle$, it is possible to calculate the free energy required to protonate a titratable group μ at a given pH value according to Eq. (20):

$$G_\mu = -RT \ln \frac{\langle x_\mu \rangle}{\left(1 - \langle x_\mu \rangle\right)} \tag{20}$$

G_μ represents a thermodynamic average over all protonation states and is the energy required to protonate group μ at a given pH and temperature, while G^i is the free energy of the specific protonation state i of the protein.

Tanford-Roxby approximation

One of the earliest attempts to calculate pK_a values of individual amino acids in proteins was made by Tanford and Roxby (1972). Later, Bashford and Karplus (1991) showed that this approach is a mean field approximation of the exact treatment. The Tanford-Roxby approximation works well for weakly interacting titratable groups, but fails if the protonation of two groups with similar pK_a is strongly coupled. It is computationally much less expensive than the exact treatment, because the summation over all possible states in Eq. (19) is avoided. As already pointed out by Tanford and Roxby (1972), the pK_a calculated by this approach depends on pH.

The Tanford-Roxby approximation assumes that the average protonation of a titratable residue depends on the average charge of all other titratable groups. The pK_a value of a titratable group in the protein is calculated iteratively. In the ith iteration, the pK_a of group μ is given by Eq. (21):

$$pK_{a,\mu}(i) = pK_{a,\mu}^{intr} - \frac{1}{RT \ln 10} \times \sum_{v=1}^{N} \left(W_{\mu v}(\langle x_\mu(i) \rangle + z_\mu^o)(\langle x_v(i) \rangle + z_v^o)\right) \tag{21}$$

where z_μ^o is the unitless formal charge of the group μ in the deprotonated form, i.e., -1 for acids and 0 for bases; $pK_{a,\mu}^{intr}$ is given by Eq. (15). The pK_a value obtained by Eq. (21) is used in Eq. (22) to calculate the protonation probability for the $(i+1)$th iteration by Eq. (22):

$$\langle x_\mu(i+1) \rangle = \frac{\exp(-\ln 10(\mathrm{pH} - pK_{a,\mu}(i)))}{1 + \exp(-\ln 10(\mathrm{pH} - pK_{a,\mu}(i)))} \tag{22}$$

The iteration proceeds until self-consistency is reached. In the initial iteration step, the pK_a values of the titratable groups are assumed to be identical with the intrinsic pK_a values $pK_{a,\mu}^{intr}$ [Eq. (15)]. Note that Eq. (21) uses $pK_{a,\mu}^{intr}$ rather than $pK_{a,\mu}$ (i), otherwise the interaction energy would accumulate during the iteration.

Reduced site approximation

In order to avoid unnecessary calculations of energies of protonation states that are unlikely to occur, Bashford and Karplus (1991) developed a method that considers

only states that contribute considerably to the summation in Eq. (19). If the pH is far away from the pH at which the particular group titrates in the protein, the group is considered to have a fixed protonation state. This reduces the number of protonation states from 2^N to 2^{N-M} if the protonation of M groups is fixed.

The groups that do not need to be considered at a specific pH value are identified as follows. The free energy for adding a proton to group μ adopts its maximum value when all other groups are also protonated; thus the maximum free energy is calculated with Eq. (23):

$$G_\mu^{\max} = G_\mu^{\text{intr}} + \sum_{\nu=1}^{N} W_{\mu\nu}\left(z_\nu^{\text{o}} + 1.0\right) \tag{23}$$

where

$$G_{\text{a},\mu}^{\text{intr}} = -RT \ln 10 \ \text{p}K_{\text{a},\mu}^{\text{intr}} \tag{24}$$

The free energy required for protonating group μ is minimal when all other titratable groups are deprotonated, i.e.

$$G_\mu^{\min} = G_\mu^{\text{intr}} + \sum_{\nu=1}^{N} W_{\mu\nu} z_\nu^{\text{o}} \tag{25}$$

At a given pH, the maximum (minimum) protonation probability of group μ is obtained from Eq. (26):

$$\langle x_\mu \rangle_{\max(\min)} = \frac{\exp\left(-\frac{G_\mu^{\max(\min)} + RT \ln 10 \ \text{pH}}{RT}\right)}{1 + \exp\left(-\frac{G_\mu^{\max(\min)} + RT \ln 10 \ \text{pH}}{RT}\right)} \tag{26}$$

Group μ is kept fixed in its protonated form if $\langle x_\mu \rangle_{\min} > 1.0 - \xi$ and in its unprotonated form if $\langle x_\mu \rangle_{\max} < \xi$; ξ is an adjustable, albeit small and positive, threshold value, which should be set to 0.05 or less. However, the protonation state of the fixed titratable groups influences the $\text{p}K_{\text{a},\mu}^{\text{intr}}$ value of the unfixed titratable group μ. The adequate correction is given by Eq. (27):

$$\text{p}K_{\text{a},\mu}^{\text{intr,correct}} = \text{p}K_{\text{a},\mu}^{\text{intr}} - \frac{1}{RT \ln 10} \sum_{\nu=1}^{N_{\text{fixed}}} W_{\mu\nu}\left(z_n^{\text{o}} + f_\nu\right) \tag{27}$$

where f_ν is either one or zero according to if group ν is fixed in the unprotonated or protonated form. The protonation probability of the titratable groups that can vary their protonation is then calculated by Eq. (19). The protonation state energies G^i [Eq. (17)] have to include now $\text{p}K_{\text{a},\mu}^{\text{intr,correct}}$ instead of $\text{p}K_{\text{a},\mu}^{\text{intr}}$.

Hybrid statistical mechanical/Tanford-Roxby algorithm

Yang et al. (1993) developed an algorithm which combines a direct evaluation of the statistical average with the Tanford-Roxby approximation. All titratable groups within a certain cut-off distance of a titratable group are treated by the direct evaluation of the statistical average, while the titratable residues outside this cut-off radius are treated with the Tanford-Roxby approximation. A cut-off distance that gave reasonable results was 7 Å (Yang et al. 1993). Alternatively, also an energy cut-off for the interaction energy $W_{\mu\nu}$ can be used (Yang et al. 1993).

In this approach, the protonation probabilities of the N titratable groups of a protein are calculated as follows. The statistical average is directly evaluated only for the j_s groups that are within a certain cut-off distance of a particular group s. The remaining $N - j_\text{s}$ groups are treated by the Tanford-Roxby approximation. The energy of the protonation substate n_s of the j_s residues in the ith iteration is given by Eq. (28):

$$\begin{aligned} G^{n_\text{s}}(i) = &\sum_{\mu=1}^{j_\text{s}} (x_\mu^{n_\text{s}} - x_\mu^{0_\text{s}}) RT \ln 10(\text{pH} - \text{p}K_\mu^{\text{intr}}) \\ &+ \frac{1}{2} \sum_{\mu=1}^{j_\text{s}} \sum_{\nu=1}^{j_\text{s}} \left(W_{\mu\nu}(x_\mu^{n_\text{s}} + z_\mu^{\text{o}})(x_\nu^{n_\text{s}} + z_\nu^{\text{o}})\right) \\ &+ \sum_{\mu=1}^{j_\text{s}} \sum_{\nu=j_\text{s}+1}^{N} \left(W_{\mu\nu}(x_\mu^{n_\text{s}} + z_\mu^{\text{o}})(\langle x_\nu(i)\rangle + z_\nu^{\text{o}})\right) \end{aligned} \tag{28}$$

The average protonation $\langle x_\text{s}(i+1)\rangle$ of group s in the $(i+1)$th iteration is calculated from Eq. (29):

$$\langle x_\text{s}(i+1)\rangle = \frac{\sum_{n_\text{s}=1}^{2^{j_\text{s}}} x_\text{s}^{n_\text{s}} \exp(-G^{n_\text{s}}(i)/RT)}{\sum_{n_\text{s}=1}^{2^{j_\text{s}}} \exp(-G^{n_\text{s}}(i)/RT)} \tag{29}$$

The same procedure is applied to all N titratable groups. Equations (28) and (29) are iterated until self-consistency is reached. In the first iteration step, the protonation probability $\langle x_\nu(1)\rangle$ of the $N - j_\text{s}$ groups that are outside the cut-off distance are calculated from Eq. (3) assuming that the $\text{p}K_\text{a}$ values of these groups are identical with their intrinsic $\text{p}K_\text{a}$ value, $\text{p}K_{\text{a},\mu}^{\text{intr}}$, obtained from Eq. (15). Alternatively, the initial $\text{p}K_\text{a}$ values can also be obtained from the Tanford-Roxby approximation or the solution $\text{p}K_\text{a}$ values of the corresponding model compounds can be used. A cut-off distance of 0 Å leads to the Tanford-Roxby approximation, while a cut-off distance of infinity leads to the exact statistical mechanical treatment. For each group, a different set of titratable groups is treated by the exact method.

A related but not identical method, the so-called cluster method, was used by Gilson (1993). In this approach, the protein is divided into clusters of coupled residues. The criterion for the clustering is the interaction energy between the titratable groups. All groups within a cluster are treated exactly, while the interaction between different clusters is treated by the Tanford-Roxby approximation. Also in this approach, the protonation probability is calculated by iterating until self-consistency is reached. Another algorithm that uses a similar idea was introduced by Karshikoff (1995).

Monte Carlo titration

Beroza et al. (1991) developed a Monte Carlo (MC) method to calculate titration curves of amino acids within proteins. With this MC method, protonation states are sampled with the probability with which they occur (importance sampling). The average protonation $\langle x_\mu \rangle$ of group μ is obtained by averaging x_μ over the sampled states. The MC titration method can also be combined with the reduced site approximation (Bashford and Karplus 1991) described above. This MC method is able to calculate titration curves of proteins as large as the photosynthetic reaction center or cytochrome *c* oxidase (Beroza et al. 1991, 1995; Lancaster et al. 1996; Kannt et al. 1998; Rabenstein et al. 1998a, b). An alternative MC titration algorithm that uses a simulated annealing protocol was proposed by Miteva et al. (1997).

Standard treatment. The initial vector $\vec{x}$ describing the protonation state of the protein is generated randomly. In a MC move the protonation of a single, randomly chosen, group μ is changed. The corresponding change in free energy ΔG_μ is obtained from Eq. (30):

$$\Delta G_\mu = \Delta x_\mu \left(\ln 10 RT (\mathrm{pH} - \mathrm{p}K_{\mathrm{a},\mu}^{\mathrm{intr}}) + \sum_{\nu=1}^{N} W_{\mu\nu} (x_\nu + z_\nu^{\mathrm{o}}) \right) \tag{30}$$

where $\Delta x_\mu = x_\mu^{\mathrm{new}} - x_\mu^{\mathrm{old}} = \pm 1$ is the change in the protonation of group μ. The new protonation state is accepted according to the Metropolis criterion, i.e. if $\Delta G \leq 0$, the protonation of group μ is always changed, if $\Delta G_\mu > 0$, the protonation of group μ is changed with probability $\exp(-\Delta G_\mu / RT)$. A MC scan is finished after N moves, i.e., after N attempts to change the protonation state. After a few hundred MC scans to reach the equilibrium, the protonation states of each scan are accumulated to evaluate the average protonation $\langle x_\mu \rangle$ of each group μ.

Treatment of strongly-coupled groups. If two titratable groups are strongly coupled, they may have two protonation states of low energy. In the two states, one proton is located either on the one or on the other group. The transition from one low energy state to the other by two subsequent MC steps involves an intermediate state, which may have a high energy. Thus the transition from one state to another may be unlikely, as depicted in Fig. 3. The problem can be avoided if the protonation of the two groups is switched simultaneously, which formally corresponds to a direct proton exchange between these two groups. The proton exchange step is also accepted according to the Metropolis criterion. If a very low dielectric constant is used for the protein ($\varepsilon \leq 2$), even three groups may couple strongly. Then the use of triple moves can help to prevent sampling problems (Rabenstein et al. 1998a).

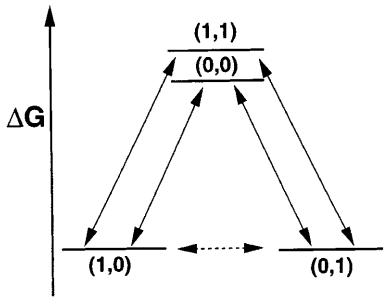

Fig. 3 Treatment of two strongly coupled groups in Monte Carlo titration calculation. If the energy barrier for the transition from state (1,0) to state (0,1) via the states (1,1) or (0,0) is too large (*solid arrows*), a Monte Carlo step is performed that simultaneously switches the protonation of both sites (*dashed arrow*)

Estimation of the statistical uncertainty. To estimate the statistical uncertainty of the MC calculation, it is necessary to calculate the number of independent data sets in the sample (Beroza et al. 1991). The correlation function $C_\mu(\tau)$ for the protonation of group μ determines a correlation time τ_μ^{corr} between approximately independent values of the protonation of group μ. It is given by Eq. (31):

$$C_\mu(\tau) = \frac{1}{T-\tau} \sum_{t=0}^{T-\tau-1} \left(x_\mu(t+\tau) x_\mu(t) - \langle x_\mu \rangle^2 \right) \tag{31}$$

where t is the time in units of one MC scan, T is the total number of scans (or the maximum time), and τ is the time variable of the correlation function. The correlation time τ_μ^{corr} is the time for which $C_\mu(\tau)$ becomes negligible (for instance $|C_\mu(\tau)| < C_\mu(0)/10$). The number of independent protonation values is $T/\tau_\mu^{\mathrm{corr}}$. If all protonation values are independent, the variance of one measurement is $C_\mu(0)$. The use of the average of $T/\tau_\mu^{\mathrm{corr}}$ independent data sets provides the standard deviation σ_μ given in Eq. (32):

$$\sigma_\mu = \sqrt{\frac{C_\mu(0)}{T/\tau_\mu^{\mathrm{corr}}}} \tag{32}$$

Titration curves for a protein with multiple conformations

Calculations that consider only a single conformation cannot be expected to predict the protonation probability correctly at each pH value, because pH-dependent conformational changes may occur and will influence the titration behavior. Even small structural changes can have a large influence on the titration behavior. Therefore $\mathrm{p}K_\mathrm{a}$ values that are far away from the pH value at which the structure was solved are often not predicted correctly. The methods described above should be able to predict the protonation probabilities of titratable groups of the protein reliably for the pH value at which the structure was solved. The inclusion of conforma-

tional changes can improve the predictive power of titration calculations and even make it possible to predict the pK_a values of titratable groups in proteins that do not titrate at the pH at which the structure was solved. The modifications of the titration algorithm that are required to account for conformational changes correctly are described in the first part of this section. In the second part, algorithms to generate different conformations of proteins are described. Such algorithms turned out to be rather complicated. In fact, most of the algorithms used so far generate protein conformations that may bias the results as discussed by Beroza and Case (1998).

Theoretical framework

In the following we assume, without loss of generality, that conformations and protonation states of a protein are independent of each other. Correlations between conformations and protonations can be considered by their probability of occurrence. Each titratable group can have multiple tautomeric forms and a protein can have many conformations. The probability of the occurrence of a particular conformation may be dependent on the protonation state of the protein and thus on the pH. A protein, which has L conformational states and N titratable groups each with n_i possible protonation forms (protonation and tautomeric forms), possesses a total number of $(L\prod_{i=1}^{N} n_i)$ states. Then the sum in Eq. (19) would have to run over all these possible states. Although a treatment allowing more than two protonation forms for each group would in principle be possible and straightforward in the theoretical framework presented here, it is not commonly used. Therefore, we refer in the following to the commonly used treatment considering only two possible protonation forms for each group. When L is the number of different conformations and N is the number of titratable groups, the number of possible states is then $\mathrm{L} \times 2^N$ if each titratable group exists in two protonation forms.

If the protein can adopt many conformations, Eq. (17) needs an additional energy term that accounts for the difference in the energies of the conformations in the reference protonation state. As long as this conformational energy term is additive, the calculation of titration curves with many conformations can be done in the same framework as the calculation of titration curves using a single conformation. The additivity is guaranteed on the basis of the LPBE. The energy of the conformational state l in the protonation state n is given by Eq. (33):

$$G^{n,l} = \sum_{\mu=1}^{N}\left((x_\mu^{n,b} - x_\mu^{0,l})RT\ln 10(\mathrm{pH} - \mathrm{p}K_{\mathrm{a},\mu}^{\mathrm{intr},l})\right) + \frac{1}{2}\sum_{\mu=1}^{N}\sum_{\nu=1}^{N}(W_{\mu\nu}^{l}(x_\mu^{n,l} + z_\mu^{\mathrm{o}})(x_\nu^{n,l} + z_\nu^{\mathrm{o}})) + \Delta G_{\mathrm{conf}}^{l} \quad (33)$$

where $\Delta G_{\mathrm{conf}}^{l} = G_{\mathrm{conf}}^{l} - G_{\mathrm{conf}}^{r}$ is the energy difference between an arbitrarily chosen, albeit fixed, reference conformation r and the actual conformation l. For the computation of ΔG_{conf} the protein must be in its reference protonation state for both conformations r and l, i.e., all titratable groups are in their uncharged protonation form.

Calculation of the conformational energies

Most studies on the effect of conformational changes of proteins neglect the change in solvation energy that arises from a conformational change. A method to account for this effect was developed in our group (Ullmann 1998) and applied to the photosynthetic reaction center (Rabenstein et al. 1998a). The energy difference between two conformations arises from electrostatic and non-electrostatic interactions. Electrostatic contributions can be calculated from the numerical solution of the LPBE. However, this numerical solution requires us to assign the atomic partial charges on discrete points of a cubic grid. This results in an artifact termed grid energy. The grid energy precludes the calculation of the conformational energies by directly evaluating the electrostatic energy difference between the two conformations connected by the thick horizontal arrow in Fig. 4. The artifact of grid energies can, however, be avoided by calculating the other three steps in the thermodynamic cycle (Ullmann et al. 1997). The energies required to transfer the protein in its reference conformation r and its conformation l from a medium with the dielectric constant of the protein (homogeneous dielectric) to a medium with the dielectric constant of water (inhomogeneous dielectric) are calculated from the numerical solution of the LPBE as given in Eq. (34). This process is depicted by the vertical arrows in Fig. 4.

$$G_{\mathrm{R}}^{l} = \frac{1}{2}\sum_{i=1}^{N_{\mathrm{p}}} q_{i,\mathrm{p}}\left(\phi_{\mathrm{p}}^{\mathrm{inhom}}(\mathbf{r}_i^{l}, q_{\mathrm{p}}) - \phi_{\mathrm{p}}^{\mathrm{hom}}(\mathbf{r}_i^{l}, q_{\mathrm{p}})\right) \quad (34)$$

The energy difference G_{R}^{l} represents the reaction field energy induced by the charges of the protein in the solvent dielectric medium. In G_{R}^{l} the grid energies as well as the Coloumb energies cancel. The atomic coordinates

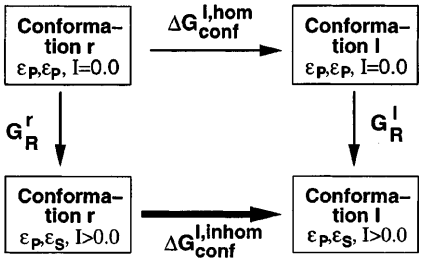

Fig. 4 Thermodynamic cycle to calculate the difference in free energy between two conformations of the same molecule

$\mathbf{r}_i^l$ for atom i refer to protein conformation l. The term $\phi_{\mathrm{p}}^{\mathrm{inhom}}(\mathbf{r}_i^l, q_{\mathrm{p}})$ denotes the value of the numerical solution of the LPBE at position $\mathbf{r}_i^l$ obtained for an inhomogeneous dielectric medium, i.e., the value of the dielectric constant is low and the ionic strength is zero within the protein, while the value of the dielectric constant is high and the ionic strength can be different from zero outside of the protein. The term $\phi_{\mathrm{p}}^{\mathrm{hom}}(\mathbf{r}_i^l, q_{\mathrm{p}})$ denotes the value of the numerical solution of the LPBE at position $\mathbf{r}_i$ obtained for a homogeneous dielectric medium, i.e., the dielectric constant adopts a low value and the ionic strength is zero everywhere. Both electrostatic fields, $\phi_{\mathrm{p}}^{\mathrm{hom}}(\mathbf{r}_i^l, q_{\mathrm{p}})$ and $\phi_{\mathrm{p}}^{\mathrm{inhom}}(\mathbf{r}_i^l, q_{\mathrm{p}})$, were obtained from all charges $q_{i,\mathrm{p}}$ of the protein in the reference protonation state, in which all titratable groups are in their uncharged protonation form. The sum runs over all N_{p} charges $q_{i,\mathrm{p}}$ of the protein. G_{R}^r is calculated analogously. The difference in solvation energy between the reference conformation r and conformation l is obtained by Eq. (35):

$$\Delta G_{\mathrm{R}}^l = G_{\mathrm{R}}^l - G_{\mathrm{R}}^r \tag{35}$$

The LPBE must be solved $2 \times L$ times for the L different protein conformations in the reference protonation state.

The energy difference along the upper arrow in Fig. 4 between the reference conformation r and conformation l in a homogeneous dielectric medium both in its reference protonation state is calculated analytically. Since the dielectric constants of the solute and the medium are equal, this energy change ΔG_{FF}^l is calculated with a conventional molecular mechanics force field using the dielectric constant of the protein ε_{P} everywhere (Brooks and Case 1993; Vásquez et al. 1994), in which also non-electrostatic interactions, i.e., van der Waals energies and bond energies, can be taken into account. The non-electrostatic solvation energy G_{NE}, which arises from non-electrostatic interactions of the molecule with its environment, is believed to be proportional to the solvent accessible surface area as shown in Eq. (36):

$$\Delta G_{\mathrm{NE}}^l = \gamma(A^l - A^r) \tag{36}$$

where A^l and A^r are the solvent accessible surfaces of the reference conformation r and conformation l respectively; γ is an empirically obtained parameter (Sitkoff et al. 1994b).

The total energy difference between conformation l and the reference conformation r is then given by Eq. (37):

$$\Delta G_{\mathrm{conf}}^l = \Delta G_{\mathrm{R}}^l + \Delta G_{\mathrm{FF}}^l + \Delta G_{\mathrm{NE}}^l \tag{37}$$

This energy difference is used in Eq. (33) to calculate the energy of the protein in conformation l and protonation state n versus conformation r and the reference protonation state. Within the framework of the LPBE, this method involves no further approximation.

Approximate methods

In principle, the calculation of intrinsic pK_{a} values $\mathrm{p}K_{\mathrm{a},\mu}^{\mathrm{intr},l}$ and the calculations of interaction energies $W_{\mu\nu}^l$ are required for each of the L different conformations. Thus the LPBE needs to be solved $2 \times L \times N$ times for the protein and also $2 \times L \times N$ times for the model compounds in order to cancel out grid artifacts properly. This leads to an enormous computational burden. In order to reduce this computational burden, You and Bashford (1995) and Beroza and Case (1996) assumed that each of the titratable groups can adopt many conformations independently. If group μ adopts M_μ different conformations, the total number of conformational states is $L = \prod_{\mu=1}^{N} M_\mu$. You and Bashford (1995) used a mean-field approximation in order to calculate the intrinsic pK_{a} values and the interaction energies. Beroza and Case (1996) and Alexov and Gunner (1997) applied a MC algorithm to determine protonation probabilities.

You and Bashford (1995) defined the intrinsic pK_{a} value as shown in Eq. (38):

$$\begin{aligned}\mathrm{p}K_{\mathrm{a},\mu}^{\mathrm{intr}} = \mathrm{p}K_{\mathrm{a},\mu}^{\mathrm{model}} - \frac{1}{\ln 10} \\ \times \Bigg[\Bigg(\ln \sum_{n=1}^{M_\mu} \exp(-\Delta G_{\mathrm{p},\mu,n}^{\mathrm{env,d}}/RT) \\ - \ln \sum_{n=1}^{M_\mu} \exp(-\Delta G_{\mathrm{p},\mu,n}^{\mathrm{env,h}}/RT) \Bigg) \\ - \Bigg(\ln \sum_{n=1}^{M_\mu} \exp(-\Delta G_{\mathrm{m},\mu,n}^{\mathrm{env,d}}/RT) \\ - \ln \sum_{n=1}^{M_\mu} \exp(-\Delta G_{\mathrm{m},\mu,n}^{\mathrm{env,h}}/RT) \Bigg) \Bigg]\end{aligned} \tag{38}$$

where the subscripts p and m denote that the energy was obtained from the protein or from the model compound respectively; n denotes a particular conformation of the titratable group μ. The superscripts h and d indicate that environmental energy ΔG^{env} was either obtained for the protonated (h) or deprotonated (d) state. The environmental energy for group μ in conformation n in the protein in its deprotonated form $G_{\mathrm{p},\mu,n}^{\mathrm{env,d}}$ is given by Eq. (39):

$$\Delta G_{\mathrm{p},\mu,n}^{\mathrm{env,d}} = E_{\mathrm{p},\mu,n} + \Delta G_{\mathrm{p},\mu,n}^{\mathrm{Born,d}} + \Delta G_{\mathrm{p},\mu,n}^{\mathrm{back,d}} \tag{39}$$

The other environmental energies are defined analogously. $E_{\mathrm{p},\mu,n}$ is the energy that is independent of the charge state of all titratable groups and may include non-electrostatic energy contributions. The terms ΔG^{Born} [Eq. (40)] and ΔG^{back} [Eq. (41)] represent the Born energy and the energy that arises from the interaction with non-titrating background charges, respectively. The meaning of the sub- and superscripts is the same as above.

$$\Delta G_{\mathrm{p},\mu,n}^{\mathrm{Born,d}} = \frac{1}{2} \sum_{i=1}^{N_{Q,\mu}} Q_{i,\mu}^{\mathrm{d}} [\phi_{\mathrm{p},\mu,n}(\mathbf{r}_i; Q_\mu^{\mathrm{d}}) - \phi_{\mathrm{unif},\mu,n}(\mathbf{r}_i; Q_\mu^{\mathrm{d}})] \tag{40}$$

$$\Delta G^{\mathrm{back,d}}_{\mathrm{p},\mu,n} = \sum_{i=1}^{N_\mathrm{p}} q_i[\phi_{\mathrm{p},\mu,n}(\mathbf{r}_i; Q^\mathrm{d}_\mu)] \tag{41}$$

$\phi_{\mathrm{unif},n}$ is the solution of the LPBE if the dielectric is uniform, i.e., the dielectric constant is set to ε_p everywhere, which is the dielectric constant of the protein. One contribution to the energy term $E_{\mathrm{p},\mu,n}$ is the change in self-energy of the titratable group μ that arises from the conformational change of the considered titratable group μ [Eq. (42)], which may change the shape of the protein and thus the dielectric boundary:

$$\Delta G^{\mathrm{selfback}}_{\mathrm{p},\mu,n} = \frac{1}{2}\sum_{i=1}^{N_\mathrm{p}} q_i\big[\phi_{\mathrm{p},\mu,n}(\mathbf{r}_i; q) - \phi_{\mathrm{unif},\mu,n}(\mathbf{r}_i; q)\big] \tag{42}$$

Equation (42) is, however, an approximation, since the self-energy term does not only depend on the conformation of group μ, but also on the conformation of all other groups.

The interaction energy between two groups μ and v is approximated by Eq. (43):

$$W_{\mu v} = \sum_{i=1}^{N_{Q,\mu}} [Q^\mathrm{h}_{\mu,i} - Q^\mathrm{d}_{\mu,i}]\big[\big\langle \phi_{\mathrm{p},v}\big(\mathbf{r}_i, Q^\mathrm{h}_v\big)\big\rangle - \big\langle \phi_{\mathrm{p},v}\big(\mathbf{r}_i, Q^\mathrm{d}_v\big)\big\rangle\big] \tag{43}$$

where the mean-field value of the electrostatic potential is

$$\big\langle \phi_{\mathrm{p},v}\big(\mathbf{r}_i, Q^\mathrm{d}_v\big)\big\rangle = \frac{\sum_{n=1}^{M_v} \phi_{\mathrm{p},v,n}\big(\mathbf{r}_i, Q^\mathrm{d}_v\big)\exp(-\Delta G^{\mathrm{env,d}}_{\mathrm{p},\mu,n}/RT)}{\sum_{n=1}^{M_v}\exp(-\Delta G^{\mathrm{env,d}}_{\mathrm{p},\mu,n}/RT)} \tag{44}$$

Owing to the averaging of the electrostatic potential, the interaction matrix W is not symmetric. It will therefore be symmetrized by $\bar{W}_{\mu v} = \frac{1}{2}\big(W_{\mu v} + W_{v\mu}\big)$ for the titration calculation. As already pointed out by You and Bashford (1995), this approximation is inadequate for groups that are in close contact. The so-obtained intrinsic pK_a values $\mathrm{p}K^{\mathrm{intr}}_{\mathrm{a},\mu}$ [Eq. (38)] and the interaction matrix $\bar{W}$ [Eq. (43)] are used in titration algorithms as described before.

With this approach, the LPBE needs to be solved $4 \times N \times \sum_{i=1}^{N} M_\mu$ times for the protein and $4 \times N \times \sum_{i=1}^{N} M_\mu$ times for all model compounds, i.e., considerably less often than the number of different conformational states that exist. The disadvantage of this approach is that correlations between protonation changes and conformational changes of closely neighbored titratable groups are not considered appropriately, as already discussed by You and Bashford (1995). Furthermore, conformational changes of the non-titratable groups are not considered.

Beroza and Case (1996) used a MC approach to include conformational flexibility into the calculation of pK_a values. They assumed that the intrinsic pK_a values $\mathrm{p}K^{\mathrm{intr,l}}_{\mathrm{a},\mu}$ and the interaction energies $W^l_{\mu v}$ do not depend on the conformation of the other titratable and non-titratable groups. Thus the LPBE needs to be solved only $2 \times N \times \sum_{i=1}^{N} M_\mu$ times for the protein and $2 \times N \times \sum_{i=1}^{N} M_\mu$ times for all model compounds. This is justifiable if the conformational changes are small, but is not generally correct, since for instance the change of the dielectric boundary influences all electrostatic energy terms (You and Bashford 1995; Beroza and Case 1996). Changes in the non-electrostatic parts of the conformational energy are neglected, but can be included. Also Beroza and Case (1996) do not consider conformational changes of non-titratable groups.

Alexov and Gunner (1997) included a non-electrostatic term in their calculations to consider differences in van der Waals interactions, torsion angle energies, and conformational changes of non-titratable groups. However, they consider only conformational changes involving hydrogen atoms. A term that accounts for changes in solvation energies is neglected in their algorithm. This is appropriate in that application only because they considered conformational changes of hydrogen atoms to which an atom radius of zero was assigned. These changes do therefore not influence the dielectric boundary and consequently also not the solvation energy of the reference state. This method cannot be applied to conformational changes in which the protein changes its shape.

Generation of different conformations

Free energy calculations using MD simulations are often used to calculate the intrinsic pK_a values and interaction energies. Furthermore, MD simulations are applied to generate different conformations (Yang and Honig 1993; Del Buono et al. 1994; Baptista et al. 1997; Sandberg and Edholm 1997; Sham et al. 1997; Zhou and Vijayakumar 1997). These approaches are the method of choice if only the protonation or redox potential of a single group is investigated (Apostolakis et al. 1996). The use of MD simulations in multiple group titrations is, however, problematic. To perform MD simulations, the protonation state of the protein has to be fixed. Consequently the results are biased towards the protonation state chosen in the MD simulation. The generation of different conformations by MD simulations bear the same problem as clearly pointed out by Wlodek et al. (1997). They performed different MD simulations of bovine pancreatic trypsin inhibitor. The protonation of the N-terminus differed in these simulations. With the equilibrated structures, titration calculations were performed. It was found that the protonated form of the N-terminus is stabilized in the structure obtained from the simulation of the protein with a protonated N-terminus, whereas the unprotonated form of the N-terminus was stabilized in the structure obtained from the simulation of the protein with the unprotonated N-terminus. This emphasizes the importance of the choice of the proper protonation state for MD simulations.

Similar problems arise if energy minimization algorithms or simulated annealing procedures are used to generate different conformations to account for the conformational variability of proteins in titration calculations (Bashford and Gerwert 1992; You and Bashford 1995; Rabenstein et al. 1998a). Rabenstein et al. (1998a) developed a method in which energy minimizations and a protonation state determination alternate iteratively. The initial protonation state was assigned according to a protonation state analysis using a dielectric constant of 4 for the protein. Fractional protonations with the corresponding values of the atomic partial charges were then assigned to the atoms of the protein and an energy minimization was started. After convergence of the minimization, the protonation state analysis was repeated but now using a dielectric constant of 2, since the energy minimization accounts for nuclear polarization. Energy minimization and protonation state determination were iterated again until self-consistency was reached. With increasing number of iterations, the titratable groups tend to adopt either a fully protonated or deprotonated state. Fractional protonations disappeared.

Beroza and Case (1996) used two local conformations for each titratable group. One side-chain conformation is the conformation taken from the crystal structure; the other was the side-chain conformation that maximizes the solvent accessibility of that side chain. With that approach they avoid the bias introduced by energy minimizations or molecular dynamics, but the physical motivation for using conformations that maximize solvent accessibility is not clear.

Antosiewicz et al. (1996b) calculated the $pK_{1/2}$ of several proteins whose structures were determined by NMR spectroscopy. The $pK_{1/2}$ values were determined for different structural models derived from NMR and arithmetically averaged to compare them with experimentally determined pK_a values. The arithmetical averaging of these $pK_{1/2}$ values has, however, no physical basis, since all considered conformations were used with the same statistical weight. The same problem exists when different crystal structures are used in titration calculations.

Several groups investigated the effect of pH on protein folding and protein denaturation. Yang and Honig (1993) and Schaefer et al. (1997) use a two-state model for protein folding. A MC procedure was used by Scheraga and co-workers (Ripoll et al. 1996; Vila et al. 1998) to generate different conformations. They used a titration procedure in order to calculate the protonation probability of the titratable groups after the MC simulation was completed. Protonation changes were not directly incorporated into the MC simulation.

The use of MC simulations has a clear advantage. The energy function does not have to be differentiable. Thus it is easily possible to introduce additional (fractional) atoms or (fractional) charges, i.e. to simulate a grant canonical ensemble. Such simulations are difficult or even impossible with MD simulations methods. Luo et al. (1998) combine the generalized Born model with a conformational search in torsion angle space. However, in their application to HIV protease, they vary only the torsion angle of two residues while they keep the rest of the protein fixed.

Proton linkage model

The proton linkage model relates the pH-induced change of the reaction free energy to the average number of protons $\Delta\bar{q}$ released from the considered molecular system, when the reaction in the investigated pH range occurs (Wyman 1964):

$$\Delta G(\mathrm{pH}_2) - \Delta G(\mathrm{pH}_1) = RT \ln 10 \int_{\mathrm{pH}_1}^{\mathrm{pH}_2} \Delta\bar{q}\,\mathrm{dpH} \qquad (45)$$

The proton linkage model relies on the assumption of a two-state system and provides, in principle, an exact method to obtain the pH dependence of the free energy. It is, however, not possible with this method to calculate absolute values for free energy changes unless a reference value at a given pH, i.e., an integration constant, is available. Detailed derivations of this model are given by several authors (Wyman 1964, 1965; Tanford 1970; Laskowski and Finkenstadt 1972). This approach can be used to determine the pH dependence of the free energy of any kind of reaction, as for instance for unfolding, association, or electron transfer reactions of proteins. It is widely used in experimental studies. In theoretical studies, it is also advantageous to use such approaches because it is for instance often not possible to determine absolute association energies or absolute protein stabilities. Therefore this approach is also frequently used in theoretical studies (Yang et al. 1993; Beroza et al. 1995; Schaefer et al. 1997; van Vlijmen et al. 1998).

Alternative approaches to protein titrations

Although we mainly focus in this review on continuum electrostatic methods using the LPBE, we shortly review alternative methods. Kesvatera et al. (1996) developed a MC method in which they used explicit ions and introduced MC steps for explicit protonation. After each tenth movement of the mobile ions, an attempt to protonate a titratable groups was made. By a self-consistent field approach, Dimitrov and Chrichton (1997) calculated pK_a values and the pH dependence of protein stability. Mehler (1996) uses a screened Coulomb potential to calculate pK_a values of proteins. This approximate approach is about 100 to 1000 times faster than finite difference LPBE approaches. Recently, several groups used the generalized Born model to calculate pK_a values of proteins and other electrostatic properties of proteins (Jayaram et al. 1998; Luo et al. 1998). These methods have the clear advantage

that a conformational search can be easily included in the calculation, as was done by Luo et al. (1998).

Applications

Reasonable agreement between calculated and measured pK_a values in proteins was obtained in several studies (Bashford and Karplus 1990, 1991; Bashford et al. 1993; Antosiewicz et al. 1994, 1996a; Tishmack et al. 1997; Forsyth et al. 1998). Also the macrodipole arising from an α-helix influences the pK_a values of titratable groups (Sitkoff et al. 1994a). Therefore it is important to include all partial charges of the protein to describe the electrostatics of a protein in detail.

Enzymatic mechanisms

Swaren et al. (1995) investigated with electrostatic methods the catalytic mechanism of a β-lactamase. They found that the residue Lys73, which is frequently discussed to be a proton acceptor for the reaction, has according to the calculation a pK_a value that is well above the physiological pH range and is thus unlikely to be involved in the reaction. Raquet et al. (1997) confirmed these results in their calculations and suggested the nearby residue Glu166 as proton acceptor. Dillet et al. (1998) investigated the pK_a values of the active site cysteines of thioredoxin of *Escherichia coli*. On the basis of their calculation they propose a reaction mechanism for the reduction of thioredoxin. Warshel and co-workers use an alternative method to investigate the energetics of enzymatic reaction (for review see Warshel and Russel 1984; Warshel and Åqvist 1991; Warshel and Papazyan 1998).

Proton transfer across membranes

Protonation events are exceptionally important for energy conducting processes in bioenergetic reactions at membranes. Therefore, pK_a calculations at these proteins have received much attention. Structures for some energy-transducing membrane proteins are available at a reasonable quality. A number of groups computed protonation probabilities for these proteins. In particular the bacterial photosynthetic reaction center (Beroza et al. 1991, 1995; Lancaster et al. 1996; Rabenstein et al. 1998a, b), bacteriorhodopsin (Bashford and Gerwert 1992; Sampogna and Honig 1994; Sandberg and Edholm 1997), and cytochrome *c* oxidase (Kannt et al. 1998) were investigated and residues that participate on proton transfer have been proposed.

Protein-protein association

The first study on the change of pK_a of proteins upon binding was done by McDonald et al. (1995) for an antigen-antibody complex of lysozyme. They found that the changes of pK_a values due to the association can be more than three pH units. The effect of ionic strength, which was also investigated by these authors, was considerably less pronounced. More recently, Gibas et al. (1997) analyzed in detail the pH dependence of the complexation of lysozyme with two different antibodies that recognize a very similar epitop. MacKerell et al. (1995) combined a continuum electrostatic titration with free energy perturbations in order to estimate the pH dependence of the binding constant of the 2′-GMP and 3′-GMP to ribonuclease T1. The influence of the redox state on association constants of cytochrome *f* and plastocyanin was studied by Soriano et al. (1997). Interestingly, the association of reduced cytochrome *f* and oxidized plastocyanin is stronger than the association of the oxidized cytochrome *f* and reduced plastocyanin. This makes sense physiologically, since the electron transfer occurs from the reduced cytochrome *f* to the oxidized plastocyanin.

Redox reactions

Muegge et al. (1996) investigated the effects of mutations on the redox potential of the special pair of the photosynthetic reaction center. Gunner et al. (1996) compared the redox potential of equivalent cofactors in the two similar branches of the photosynthetic reaction center. The protein environment shifts the redox potential of the cofactors differently in both branches. Because only one of the two branches is electron transfer active, they suggested that the difference of the redox potentials of the two branches is partially responsible for this observation. Gunner and Honig (1991) determined the redox potential of the four different hemes of the cytochrome subunit of the *Rhodopseudomonas viridis* photosynthetic reaction center. They considered in their study the average protonation of the titratable groups. Soares et al. (1997) investigated the redox-Bohr effect in cytochrome c_3 by continuum electrostatic methods. They performed a titration on cytochrome c_3 in different redox states and were able to identify residues that are probably responsible for the redox-Bohr effect. However, Gunner and Honig (1991) and Soares et al. (1997) neglected both the dependence of the redox state and protonation state of the protein on the solution redox potential.

Hydrogen exchange

Electrostatic methods have been used for quite a while to interpret experimental data for hydrogen exchange. Deleiere et al. (1987) applied the model developed by Tanford and Kirkwood (1957) for that purpose. Recently, Fogolari et al. (1998) estimated the pK_a shift of backbone amides and investigated the effect on the hydrogen exchange rates of small peptides. Multiply ionized states of backbone atoms were neglected in these calculations, since the depronation of backbone atoms are rare

and short-lived events. All ionization states of titratable groups have, however, been considered explicitly.

Limitations and future developments

Techniques for including of conformational flexibility in titration calculations are still under development. The main problem remains to generate appropriate molecular ensembles at a given pH and temperature. The methods applied so far have several shortcomings. For instance, the chosen conformational ensemble does not necessarily represent the protein conformational space at the investigated pH range adequately. No conformational variation of the protein backbone is currently allowed during the computational titration. Only preselected conformations can be used. The preselected conformations should be properly partioned, i.e., none of the conformations should be entropically or energetically favored to avoid double counting, but this can not be guaranteed generally. Apparently, molecular dynamics or energy minimization approaches are not suitable to reach that goal because correlations between the protonation state and the molecular conformations occur. During a molecular dynamics simulation, it is impossible to change the protonation pattern discontinuously. A complete free energy simulation in which the protonation states are changed continuously is too time consuming for proteins that have many titratable groups. This problem does not occur with MC dynamics. An efficient off-lattice MC dynamics method for proteins was recently developed in our group (Knapp and Irgens-Defregger 1991; Hoffmann and Knapp 1996a, b, 1997; Sartori et al. 1998; Hoffmann et al. 1999), and we are now working on combining this MC dynamics with MC titration. MC dynamics will also solve another problem which appears with structural relaxation by energy minimization. In contrast to energy minimization, molecular dynamics and MC dynamics are able to overcome energy barriers. This is often necessary, even if only apparently small conformational changes are involved.

Acknowledgements This work was supported by the Deutsche Forschungsgemeinschaft SFB 312, Project D7. G.M.U. thanks the Boehringer Ingelheim Fonds for a fellowship. We thank Dr. Bashford, Dr. Ingo Muegge, Björn Rabenstein and Dr. Spassor for helpful discussions und Michael Rempel for his help with Fig. 2.

References

Alexov EG, Gunner MR (1997) Incorporating protein conformational flexibility into the calculation of pH dependent protein properties. Biophys J 74: 2075–2093

Antosiewicz J, McCammon JA, Gilson MK (1994) Prediction of pH-dependent properties of proteins. J Mol Biol 238: 415–436

Antosiewicz J, Briggs JM, Elcock AM, Gilson MK, McCammon JA (1996a) Computing ionization states of protein with a detailed charge model. J Comput Chem 17: 1633–1644

Antosiewicz J, McCammon JA, Gilson MK (1996b) The determinants of pK_as in proteins. Biochemistry 35: 7819–7833

Apostolakis J, Muegge I, Ermler U, Fritzsch G, Knapp E (1996) Free energy computations on the shift of the special pair redox potential: mutants of the reaction center of *Rhodobacter sphaeroides*. J Am Chem Soc 118: 3743–3752

Baerends EJ, Gritsenko OV (1997) A quantum chemical view of density functional theory. J Phys Chem A 101: 5383–5403

Baptista AM, Martel PJ, Peters SB (1997) Simulation of protein conformational freedom as a function of pH: constant-pH molecular dynamics using implicit titration. Proteins 27: 523–544

Bashford D (1991) Electrostatic effects in biological molecules. Curr Opin Struct Biol 1: 175–184

Bashford D, Gerwert K (1992) Electrostatic calculations of the pK_a values of ionizable groups in bacteriorhodopsin. J Mol Biol 224: 473–486

Bashford D, Karplus M (1990) pK_as of ionizable groups in proteins: atomic detail from a continuum electrostatic model. Biochemistry 29: 10219–10225

Bashford D, Karplus M (1991) Multiple-site titration curves of proteins: an analysis of exact and approximate methods for their calculations. J Phys Chem 95: 9557–9561

Bashford D, Case DA, Dalvit C, Tennant L, Wright PE (1993) Electrostatic calculations of side-chain pK_a values in myoglobin and comparisation with NMR data for histidines. Biochemistry 32: 8045–8056

Beroza P, Case DA (1996) Including side chain flexibility in continuum electrostatic calculations of protein titration. J Phys Chem 100: 20156–20163

Beroza P, Case DA (1998) Calculation of proton binding thermodynamics in proteins. Methods Enzymol 295: 170–189

Beroza P, Fredkin DR (1996) Calculation of amino acid pK_as in a protein from a continuum electrostatic model: methods and sensitivity analysis. J Comput Chem 17: 1229–1244

Beroza P, Fredkin DR, Okamura MY, Feher G (1991) Protonation of interacting residues in a protein by a Monte Carlo method: application to lysozyme and the photosynthetic reaction center. Proc Natl Acad Sci USA 88: 5804–5808

Beroza P, Fredkin DR, Okamura MY, Feher G (1995) Electrostatic calculation of amino acid titration and electron transfer $Q_A^- Q_B \rightarrow Q_A Q_B^-$ in the reaction center. Biophys J 68: 2233–2250

Breneman CN, Wiberg KB (1990) Determining atom-centered monopoles from molecular electrostatic potentials. Need for high sampling density in formamide conformational analysis. J Comput Chem 11: 361–373

Brooks CL, Case DA (1993) Simulation of peptide conformational dynamics and thermodynamics. Chem Rev 93: 2487–2502

Chandra AK, Goursot A (1996) Calculation of proton affinities using density functional procedures: a critical study. J Phys Chem 100: 11596–11599

Chen JL, Noodleman L, Case D, Bashford D (1994) Incorporating solvation effects into density functional electronic structure calculations. J Phys Chem 98: 11059–11068

Cramer WA, Knaff DB (1991) Energy transduction in biological membranes. Springer, New York

Daune M (1997) Molekulare biophysik. Vieweg, Braunschweig

Del Buono GS, Figueirisco FE, Levy R (1994) Intrinsic pK_as of ionizable residues in proteins: an explicit solvent calculation for lysozyme. Proteins 20: 85–97

Deleiere M, Dobson CM, Karplus M, Poulsen FM, States DJ, Wedin RE (1987) Electrostatic effects and hydrogen exchange behaviour in proteins. J Mol Biol 196: 111–130

Demchuck E, Wade RC (1996) Improving the continuum dielectric approach to calculating pK_as of ionizable groups in proteins. J Phys Chem 100: 17373–17387

Dillet V, Dyson HJ, Bashford D (1998) Calculations of electrostatic interactions and pK_as in the active site of *Escherichia coli* thioredoxin. Biochemistry 37: 10298–10306

Dimitrov RA, Crichton RR (1997) Self-consistent field approach to protein structure and stability. I: pH dependence of electrostatic contributions. Proteins 27: 576–596

Fogolari F, Esposito G, Viglino P, Briggs JM, McCammon JA (1998) pK_a shift effects on backbone amide base-catalyzed hydrogen exchange rates in peptides. J Am Chem Soc 120: 3735–3738

Forsyth WR, Gilson MK, Antosiewicz J, Jaren OR, Robertson AD (1998) Theoretical and experimental analysis of ionization equilibria in ovomucoid third domain. Biochemistry 37: 8643–8652

Gibas CJ, Subramaniam S (1996) Explicit solvent models in protein pK_a calculations. Biophys J 71: 138–147

Gibas CJ, Subramaniam S, McCammon JA, Braden BC, Poljak RJ (1997) pH dependence of antibody/lysozyme complexation. Biochemistry 36: 15599–15614

Gilson MK (1993) Multiple-site titration and molecular modelling: Two rapid methods for computing energies and forces for ionizable groups in proteins. Proteins 15: 266–282

Gilson MK (1995) Theory of electrostatic interactions in macromolecules. Curr Opin Struct Biol 5: 216–223

Gilson MK, Given JA, Bush BL, McCammon JA (1997) The statistical thermodynamic basis for computation of binding affinities: a critical review. Biophys J 72: 1047–1069

Gunner MR, Honig B (1991) Electrostatic control of midpoint potentials in the cytochrome subunit of *Rhodopseudomonas viridis* reaction center. Proc Natl Acad Sci USA 88: 9151–9155

Gunner MR, Nicholls A, Honig B (1996) Electrostatic potentials in *Rhodopseudomonas viridis* reaction centers: implications for the driving force and directionality of the electron transfer. J Phys Chem 100: 4277–4291

Gunsteren WF van, Berendsen HJC (1990) Computer simulation of molecular dynamics: methodology, applications, and perspectives in chemistry. Angew Chem Int Ed Engl 29: 992–1023

Harvey SC (1989) Treatment of electrostatic effects in macromolecular modelling. Proteins 5: 78–92

Hoffmann D, Knapp EW (1996a) Polypeptide folding with off-lattice Monte Carlo dynamics: the method. Eur Biophys J 24: 387–403

Hoffmann D, Knapp EW (1996b) Protein dynamics with off-lattice Monte Carlo moves. Phys Rev E 53: 4221–4224

Hoffmann D, Knapp E.-W (1997) Folding pathways of a helix-turn-helix model protein. J Phys Chem B 101: 6734–6740

Hoffmann D, Washio T, Gessler K, Jacob J (1999) Tackling concrete problems in molecular biophysics using Monte Carlo and related methods: glycosylation, folding, solvation. In: Grassberger P, Barkema GT, Nadler W (eds) Monte Carlo approach to biopolymers and protein folding. World Scientific, Singapore, pp 153–170

Holleman AF, Wiberg E (1964) Lehrbuch der anorganischen Chemie. de Gruyter, Berlin

Holst MJ, Saied F (1995) Numerical solution of the nonlinear Poisson-Boltzmann equation: developing more robust and efficient methods. J Comput Chem 16: 337–364

Holst MJ, Kozack RE, Saied F, Subramiam S (1994) Treatment of electrostatic effect in proteins: multigrid-based Newton iterative method for solution of the full nonlinear Poisson-Boltzmann equation. Proteins 18: 231–245

Honig B, Nicholls A (1995) Classical electrostatics in biology and chemistry. Science 268: 1144–1149

Jayaram B, Liu Y, Beveridge DL (1998) A modification of the generalized Born theory for improved estimates of solvation energies and p*K* shifts. J Chem Phys 109: 1465–1471

Juffer AH, Argos P, Vogel HJ (1997) Calculating acid-dissociation constants of proteins using the boundary element method. J Phys Chem B 101: 7664–7673

Kaim W, Schwederski B (1995) Bioinorganic chemistry: inorganic elements in the chemistry of life. Wiley, New York

Kallies B, Mitzner R (1997) pK_a values of amines in water from quantum mechanical calculations using a polarized dielectric continuum representation of the solvent. J Phys Chem B 101: 2959–2967

Kannt A, Lancaster CRD, Michel H (1998) The coupling of electron transfer and proton translocation: electrostatic calculations on *Paracoccus denitrificans* cytochrome *c* oxidase. Biophys J 74: 708–721

Karplus M, Petsko GA (1990) Molecular dynamics simulations in biology. Nature 347: 631–639

Karshikoff A (1995) A simple algorithm for the calculation of multiple site titration curves. Protein Eng 8: 243–248

Kesvatera T, Jönsson B, Thulin E, Linse S (1996) Measurement and modelling of sequence-specific pK_a values of lysine residues in calbindin D_{9k}. J Mol Biol 259: 828–839

Kirkwood JG (1934) Theory of solution of molecules containing widely separated charges with special application to zwitterions. J Chem Phys 2: 351–361

Knapp E, Irgens-Defregger A (1991) Long time dynamics of proteins: an off-lattice Monte Carlo method. In: Harms U (ed) Supercomputer and chemistry, vol 2. Springer, Berlin Heidelberg New York, pp 83–106

Kollman PA (1993) Free energy calculations: applications to chemical and biochemical phenomena. Chem Rev 93: 2395–2417

Lancaster CR, Michel H, Honig B, Gunner MR (1996) Calculated coupling of electron and proton transfer in the photosynthestic reaction center of *Rhodopseudomonas viridis*. Biophys J 70: 2469–2492

Laskowski M, Finkenstadt WR (1972) Study of protein-protein and of protein-ligand interaction by potentiometric methods. Methods Enzymol 26: 193–227

Li J, Fischer CL, Chen JL, Bashford D, Noodleman L (1996) Calculation of redox potentials and pK_a values of hydrated transition metal cations by a combined density functional and continuum dielectric theory. J Phys Chem 96: 2855–2866

Li J, Nelson MR, Peng CY, Bashford D, Noodleman L (1998) Incorporating protein environments in density functional theory: a self-consistent reaction field calculation of redox potentials of [2Fe2S] clusters in ferredoxin and phthalate dioxygenase reductase. J Phys Chem A 102: 6311–6324

Lim C, Bashford D, Karplus M (1991) Absolute pK_a calculations with continuum dielectric methods. J Phys Chem 95: 5610–5620

Lowe JP (1993) Quantum chemistry. Academic Press, San Diego

Luo R, Martha S, Head JM, Gilson MK (1998) pK_a shifts in small molecules and HIV protease: electrostatics and conformation. J Am Chem Soc 120: 6138–6146

MacKerell AD, Sommer MS, Karplus M (1995) pH dependence of binding reactions from free energy simulations and macroscopic continuum electrostatic calculations: application to 2′GMP/3′GMP binding to ribonuclease T_1 and implications for catalysis. J Mol Biol 247: 774–807

Madura JD, Davis ME, Gilson MK, Wade RC, Luty BA, McCammon JA (1994) Biological applications of electrostatic calculations and Brownian dynamics. Rev Comput Chem 5: 229–267

McDonald SM, Willson RC, McCammon JA (1995) Determination of the pK_a values of titratable groups of an antigen-antibody complex, HyHEL-5-hen egg lysozyme. Protein Eng 8: 915–924

Mehler EL (1996) Self-consistent, free energy based approximation to calculate pH dependent electrostatic effects in proteins. J Phys Chem 100: 16006–16018

Merril GN, Kass SR (1996) Calculated gas-phase acidities using density functional theory: is it reliable? J Phys Chem 100: 17465–17471

Miteva M, Demirev PA, Karshikof AD (1997) Multiply-protonated protein ions in the gas phase: calculation of the electrostatic interactions between charged sites. J Phys Chem B 101: 9645–9650

Moore GR, Pettigrew GW (1990) Cytochromes *c*: evolutionary, structural and physicochemical aspects. Springer, Berlin Heidelberg New York

Moult J (1992) Electrostatics. Curr Opin Struct Biol 223: 223–229

Mousca J-M, Chen JL, Noodleman L, Bashford D, Case DA (1995) Density functional/Poisson-Boltzmann calculations of redox potentials for iron-sulfur clusters. J Am Chem Soc 116: 11989–11914

Muegge I, Apostolakis J, Ermler U, Fritsch G, Lubitz W, Knapp EW (1996) Shift of the special pair redox potential: electrostatic computations of mutants of the reaction center from *Rhodobacter sphaeroides*. Biochemistry 35: 8359–8370

Naray-Szabó G, Ferenczy GG (1995) Molecular electrostatics. Chem Rev 95: 829–847

Nozaki Y, Tanford C (1967) Examination of titration behavior. Methods Enzymol 11: 715–734

Oberoi H, Allewell N (1993) Multigrid solution of the nonlinear Poisson-Boltzmann equation and calculation of titration curves. Biophys J 65: 48–55

Potter MJ, Gilson MK, McCammon JA (1994) Small molecule pK_a prediction with continuum electrostatics calculations. J Am Chem Soc 116: 10298–10299

Rabenstein B, Ullmann GM, Knapp EW (1998a) Calculation of protonation patterns in proteins with conformational relaxation – application to the photosynthetic reaction center. Eur Biophys J 27: 628–637

Rabenstein B, Ullmann GM, Knapp EW (1998b) Energetics of the electron transfer and protonation reactions of the quinones in the photosynthetic reaction center of *Rhodopseudomonas viridis*. Biochemistry 37: 2488–2495

Rao STF, Shaffie CY, Satyshur KA, Stockman BJ, Markley JL, Sundaralingam M (1993) Structure of the oxidized long chain flavodoxin from *Anabaena* 7120 at 2 Å resolution. Prot Sci 1: 1413–1427

Raquet X, Lounnas V, Lamotte-Brasseur J, Frere JM, Wade RC (1997) pK_a Calculations for class A beta-lactamases: methodological and mechanistic implications. Biophys J 73: 2416–2426

Richardson WH, Peng C, Bashford D, Noodleman L, Case DA (1997) Incorporating solvation effects into density functional theory: calculation of absolute acidities. Int J Quantum Chem 61: 207–217

Ripoll DR, Vorobjev YN, Liwo A, Vila JA, Scheraga HA (1996) Coupling between folding and ionization equilibria: effects of pH on the conformational preferences of polypeptides. J Mol Biol 264: 770–783

Sampogna RV, Honig B (1994) Environmental effects on the protonation states of the active site residues in bacteriorhodopsin. Biophys J 66: 1341–1352

Sandberg L, Edholm O (1997) pK_a calculations along a bacteriorhodopsin molecular dynamics trajectory. Biophys Chem 65: 189–204

Sartori F, Melchers B, Böttcher H, Knapp EW (1998) An energy function for dynamics simulations of polypeptides in torsion angle space. J Chem Phys 108: 8264–8276

Schaefer M, Karplus M (1996) A comprehensive analytical treatment of continuum electrostatics. J Phys Chem 100: 1578–1599

Schaefer M, Sommer M, Karplus M (1997) pH-dependence of protein stability: absolute electrostatic free energy differences between conformations. J Phys Chem B 101: 1663–1683

Schaefer M, Bartels C, Karplus M (1998) Solution conformations and thermodynamics of structured peptides: molecular dynamics simulation with an implicit solvation model. J Mol Biol 284: 835–848

Sham YY, Chu ZT, Warshel A (1997) Consistent calculation of pK_a's of ionizable residues in proteins: semi-microscopic and microscopic approaches. J Phys Chem B 101: 4458–4472

Sharp KA (1994) Electrostatic interactions in macromolecules. Curr Opin Struct Biol 4: 234–239

Sharp KA, Honig B (1990) Electrostatic interactions in macromolecules. Annu Rev Biophys Biophys Chem 19: 301–332

Simonson T, Brooks CL (1996) Charge separation and the dielectric constant of proteins: insights from molecular dynamics. J Am Chem Soc 118: 8452–8458

Simonson T, Perahia D (1995a) Internal and interfacial dielectric properties of cytochrome *c* from molecular dynamics in aqueous solution. Proc Natl Acad Sci USA 92: 1082–1086

Simonson T, Perahia D (1995b) Microscopic dielectric properties of cytochrome *c* from molecular dynamics simulation in aqueous solution. J Am Chem Soc 117: 7987–8000

Simonson T, Perahia D, Bricogne G (1992) Intramolecular dielectric screening in proteins. J Mol Biol 218: 859–886

Sitkoff D, Lockhart DJ, Sharp KA, Honig B (1994a) Calculation of electrostatic effects at the amino terminus of an α-helix. Biophys J 67: 2251–2260

Sitkoff D, Sharp KA, Honig B (1994b) Accurate calculation of hydration free energies using macroscopic solvent models. J Phys Chem 98: 1978–1988

Sklenar H, Eisenhaber F, Poncin M, Lavery R (1990) Including solvent and counterion effects in the force fields of macromolecular mechanics: the field integrated electrostatic approach (FIESTA). In: Beveridge DL, Lavery R (eds) Theoretical biochemistry & molecular biophysics. Adenine Press, New York, pp 317–336

Soares CM, Martel PJ, Carrondo MA (1997) Theoretical studies on the redox-Bohr effect in cytochrome c_3 from *Desulfovibrio vulgaris* Hildenborough. J Biol Inorg Chem 2: 714–727

Soriano GM, Cramer WA, Krishtalik LI (1997) Electrostatic effects on electron-transfer kinetics in the cytochrome *f*-plastocyanin complex. Biophys J 73: 3265–3276

Still WC, Tempczyk A, Hawley RC, Hendrickson T (1990) Semianalytical treatment of solvation for molecular mechanics and dynamics. J Am Chem Soc 112: 6127–6129

Swaren P, Maveyraud L, Guillet V, Masson J-M, Mourey L, Samama J-P (1995) Electrostatic analysis of TEM1 b-lactamase: effect of substrate binding, steep potential gradients and consequences of site-directed mutations. Structure 3: 603–613

Szabo A, Ostlund NS (1989) Modern quantum chemistry. Dover, New York

Tanford C (1962) The interpretation of hydrogen ion titration curves of proteins. Adv Protein Chem 17: 69–165

Tanford C (1970) Protein denaturation. Part C: theoretical models for the mechanisms of denaturation. Adv Protein Chem 25: 1–95

Tanford C, Kirkwood JG (1957) Theory of protein titration curves. J Am Chem Soc 79: 5333–5347

Tanford C, Roxby R (1972) Interpretation of protein titration curves. Application to lysozyme. Biochemistry 11: 2192–2198

Tanokura M (1983) ^{1}H-NMR study on the tautomerism of the imidazole ring of histidine residues. I. Microscopic pK values and molar ratios of tautomers in histidine-containing peptides. Biochim Biophys Acta 742: 576–585

Tishmack PA, Bashford D, Harms E, Etten RLV (1997) Use of ^{1}H NMR spectroscopy and computer simulations to analyze histidine pK_a changes in a protein tyrosine phosphatase: experimental and theoretical determination of electrostatic properties in a small protein. Biochemistry 39: 11984–11994

Topol IA, Tawa GJ, Burt SK, Rashin AA (1997) Calculation of absolute and relative acidities of substituted imidazoles in aqueous solvent. J Phys Chem A 101: 10075–10081

Ullmann GM (1998) Simulation and analysis of docking and molecular dynamics of electron-transfer protein complexes. PhD thesis, Freie Universität Berlin

Ullmann GM, Muegge I, Knapp EW (1996) Shifts of the special pair redox potential of mutants of *Rhodobacter sphaeroides* calculated with Delphi and Charmm energy functions. In: Michel-Beyerle EM (ed): The reaction centers of photosynthetic bacteria. Structure and dynamics. Springer, Berlin Heidelberg New York, pp 143–155

Ullmann GM, Knapp EW, Kostić NM (1997) Computational simulation and analysis of the dynamic association between plastocyanin and cytochrome *f*. Consequences for the electron-transfer reaction. J Am Chem Soc 119: 42–52

Vásquez M, Némethy G, Scheraga HA (1994) Conformational energy calculations on polypeptides and proteins. Chem Rev 94: 2183–2239

Vila JA, Ripoll DR, Vorobjev YN, Scheraga HA (1998) Computation of the structure-dependent pK_a shifts in a polypentapeptide of the poly[fv(IPGVG), fE(IPGEG)] family. J Phys Chem B 102: 3065–3067

Vilaiño T, Fiol S, Armesto XL, Brandariz I, Saraste de Vicente ME (1997) Effect of ionic strength on the protonation of various

amino acids analysed by the mean sperical approximation. J Chem Soc Faraday Trans 93: 413–417

Vlijmen HWT van, Curry S, Schaefer M, Karplus M (1998) Titration calculations of foot-and-mouth disease virus capsids and their stabilities as a function of pH. J Mol Biol 275: 295–308

Warshel A, Åqvist J (1991) Electrostatic energy and macromolecular function. Annu Rev Biophys Biophys Chem 20: 267–298

Warshel A, Papazyan A (1998) Electrostatic effects in macromolecules: fundamental concepts and practical modeling. Curr Opin Struct Biol 8: 211–217

Warshel A, Russel ST (1984) Calculations of electrostatic interaction in biological systems and in solution. Q Rev Biophys 17: 283–422

Weast RC (ed) (1986) CRC handbook of physics and chemistry. CRC Press, Boca Raton

Wilson GS (1983) Electrochemical studies of porphyrin redox reactions as cytochrome models. Bioelectrochem Bioenerg 1: 172–179

Wlodek ST, Antosiewicz J, McCammon JA (1997) Prediction of titration properties of structures of a protein derived from molecular dynamics trajectories. Protein Sci 6: 373–382

Wyman J (1964) Linked functions and reciprocal effects in hemoglobin: a second look. Adv Protein Chem 19: 223–286

Wyman J (1965) The binding potential, a neglected linkage concept. J Mol Biol 11: 631–644

Yagil G (1967) The proton dissociation constant of pyrrole, indole and related compounds. Tetrahedron 23: 2855–2861

Yang A-S, Honig B (1993) On the pH dependence of protein stability. J Mol Biol 231: 459–474

Yang A-S, Gunner MR, Sompogna R, Honig B (1993) On the calculation of pK_as in proteins. Proteins 15: 252–265

You T, Bashford D (1995) Conformation and hydrogen ion titration of proteins: a continuum electrostatic model with conformational flexibility. Biophys J 69: 1721–1733

Zauhar RY, Varnek A (1996) A fast and space-efficient boundary element method for computing electrostatic and hydration effects in large molecules. J Comput Chem 17: 864–877

Zhou H-X, Vijayakumar M (1997) Modeling of protein conformational fluctuations in pK_a predictions. J Mol Biol 267: 1002–1011

Ziegler T (1991) Approximate density functional theory as a practical tool in molecular energetics and dynamics. Chem Rev 91: 651–667