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Protonation of interacting residues in a protein by a Monte Carlo method: Application to lysozyme and the photosynthetic reaction center of *Rhodobacter sphaeroides*

(bacterial photosynthesis/protein electrostatics/Poisson–Boltzmann/electron transfer/proton transfer)

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ABSTRACT **We used Monte Carlo methods to treat the statistical problem of electrostatic interactions among many titrating amino acids and applied these methods to lysozyme and the photosynthetic reaction center of *Rhodobacter sphaeroides*, including all titrating sites. We computed the average protonation of residues as a function of pH from an equilibrium distribution of states generated by random sampling. Electrostatic energies were calculated from a finite difference solution to the linearized Poisson–Boltzmann equation using the coordinates from solved protein structures. For most calculations we used the Metropolis algorithm to sample protonation states; for strongly coupled sites, we substantially reduced sampling errors by using a modified algorithm that allows multiple site transitions. The Monte Carlo method agreed with calculations for a small test system, lysozyme, for which the complete partition function was calculated. We also calculated the pH dependence of the free energy change associated with electron transfer from the primary to the secondary quinone in the photosynthetic reaction center. The shape of the resulting curve agreed fairly well with experiment, but the proton uptake from which the free energy was calculated agreed only to within a factor of two with the observed values. We believe that this discrepancy resulted from errors in the individual electrostatic energy calculations rather than from errors in the Monte Carlo sampling.**

Electrostatic interactions in proteins are important for protein structure and function. The largest contribution to the electrostatic potential within a protein arises from protonatable amino acids that can carry a net charge. The problem of determining the average charges on protonatable residues can be separated into two parts. (*i*) The energies of protonation of the individual amino acids and the interaction energies between pairs of charged residues must be calculated. Much progress along these lines has been made (1–4). (*ii*) The average protonation of each residue must be determined from the electrostatic energies. Since the protonation of a site depends on the protonation of all other sites (in a typical protein there may be hundreds of titrating sites), an exact statistical calculation becomes too time consuming for more than ≈25 titrating residues. In this paper we present a Monte Carlo method to solve the statistical problem of finding the protonation of many interacting protonatable residues.

Previous methods used to solve this problem can handle only a small number of sites or are inaccurate. Exact values of average protonations calculated from a partition function work well when the number of sites is below ≈25, and the reduced-site approximation can treat twice as many sites for some systems (5). The Tanford–Roxby approximation (6) ignores fluctuations in the protonation of residues and has been shown to be inaccurate for strongly interacting titrating residues (5).

We used a Monte Carlo technique for determining the protonation of many interacting sites as a function of pH. Our method, which employs a finite difference algorithm to calculate electrostatic energies, can treat hundreds of interacting titrating residues. A Monte Carlo sampling of protonation states was employed by Antosiewiez and Porschke (7) to determine dipole moments in α-chymotrypsin.

We present a formalism for multiple-site titration, followed by a description of the Monte Carlo approach to the problem, and of a sampling technique that reduces sampling errors. We tested the accuracy of the method against an exact solution for a small test system (lysozyme, in which only 21 of the 32 sites were allowed to titrate). We applied the technique to two systems: (*i*) the titration of all 32 sites in lysozyme and (*ii*) the pH dependence of the free energy of electron transfer between the primary and secondary quinones (Q_A and Q_B) in the photosynthetic reaction center of *Rhodobacter (Rb.) sphaeroides*, taking into account all 172 titratable residues. Fair agreement between the calculated and observed values was obtained. Further approaches to improve the agreement between calculations and experiments are discussed.

Multiple Site Titration

The titration curve of a residue in a protein is obtained from the pH dependence of its average protonation, $\langle x_i \rangle$, where x_i is the number of protons bound to the ith titratable site (x_i = 0 or 1). We represent the protonation state of a protein with N titrating sites by a vector with N components, $\mathbf{x} = (x_1, x_2, \ldots, x_N)$. The free energy associated with protonation is

$$G_p(\mathbf{x}) = \sum_{i=1}^{N} x_i(\varepsilon_i - \mu_{H^+}) + \frac{1}{2}\sum_{\substack{i,j=1 \\ i\neq j}}^{N} W_{ij}q_iq_j, \qquad [1]$$

where ε_i is the intrinsic free energy required to protonate site i when all other titrating sites are maintained neutral, μ_{H^+} is the hydrogen ion chemical potential in the solution, q_i is the charge on site i, $q_i = q_i^0 + x_i$, where q_i^0 is the charge of site i in the unprotonated state, and W_{ij} is the electrostatic interaction energy between sites i and j when both are charged.

The intrinsic free energy ε_i of protonating a site is a function of the protein environment, including solvation, background charges in the protein (fixed charges and permanent dipoles), polarization at the protein–solvent interface, and the screening effects of salt. It is related to the intrinsic pK by

$$\varepsilon_i - \mu_{H^+} = k_BT[\ln 10](\mathrm{pH} - \mathrm{pK}_i^0), \qquad [2]$$

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where pK_i^0 is the pK of a residue when all other protonatable residues are in their neutral state, k_B is Boltzmann's constant, and T is the temperature.

The average protonation of a site is given by

$$\langle x_i \rangle = \frac{\sum_{\mathbf{x}} x_i e^{-G(\mathbf{x})/k_B T}}{Z}, \quad [3]$$

where $Z = \Sigma_{\mathbf{x}} E^{-G(x)/k_B T}$ is the partition function (8), and the sum is over the 2^N different protonation states (canonical ensemble). This average calculated at various values of pH yields a titration curve, and the pK of each site i in the protein is defined as the pH for which $\langle x_i \rangle = 1/2$.

Monte Carlo Method

The number of terms in the partition function and hence the thermal average $\langle x_i \rangle$ grows exponentially with the number of sites; for more than ≈25 sites the calculation of the sum (Eq. 3) is too time consuming for even the fastest present-day computers. Nevertheless, the average behavior of such a system can be obtained by a Monte Carlo (i.e., random) sampling of the 2^N protonation states.

An especially efficient Monte Carlo method, called importance sampling (9), samples protonation states with probability given by the normalized Boltzmann factor, $P_{\mathbf{x}} = \exp[-G(\mathbf{x})/k_B T]/Z$. The statistically most important states (i.e., the low-energy states) are sampled with the correct statistical distribution, while the unlikely states are avoided. Thus, the number of sampled states is small, compared with the total number of accessible states. The average protonation of a residue $\langle x_i \rangle$ is approximated by averaging x_i over the sampled states.

Standard Treatment. In most of our calculations we used the Metropolis algorithm to sample protonation states (10). Starting from a randomly chosen initial protonation state, successive states are generated by a stochastic process: Choose a site, i, randomly, all sites being equally likely. Compute the change in energy, ΔG, if the protonation of site i is changed:

$$\Delta G = \Delta x_i \left(\varepsilon_i - \mu_{H^+} + \sum_{j \neq i}^{N} W_{ij} q_j \right), \quad [4]$$

where $\Delta x_i = \pm 1$ is the change in protonation of site i. If $\Delta G \leq 0$, change the protonation of site i; if $\Delta G > 0$, change the protonation with probability $e^{-\Delta G/k_B T}$.

Strongly Coupled Sites. The Metropolis algorithm may be inefficient for sampling strongly interacting sites because the protonation of only *one* site is allowed to change between successively sampled states. This restriction may lead to energy barriers between states of low energy, causing the Monte Carlo trajectory to be trapped in a local minimum, thereby reducing sampling efficiency.

Consider, for example, a protein containing two strongly interacting cationic sites (charged in their protonated states) with equal intrinsic pK values. If the pH value is below the intrinsic pK values of the sites, then the states (1,1) and (0,0) are energetically unfavorable [the (1,1) state because of Coulomb repulsion and the (0,0) state because pH < pK^0] compared to the singly protonated states (1,0) and (0,1). Because the two low-energy states are isoenergetic, the sampling algorithm should choose them with equal frequency. However, a long computation is required to obtain correct statistics, because transitions between the two equally probable low-energy states occur rarely. This is a feature of the Metropolis algorithm, which allows only one of the sites to change per sampling step. Therefore, two transitions are needed to change from (0,1) to (1,0) (see solid line in Fig. 1), requiring an unlikely visit to a high-energy intermediate state (0,0) or (1,1).

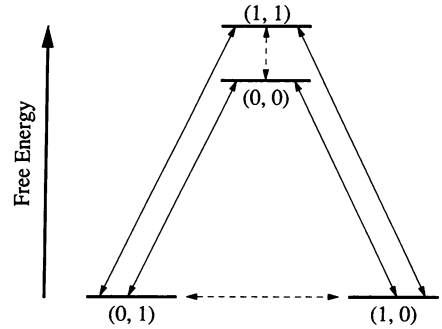

FIG. 1. Energy diagram showing the four protonation states of two strongly interacting sites. The two low-energy states with one proton [(0,1) and (1,0)] are degenerate and, therefore, should be sampled with equal frequency. Transitions allowed by conventional Metropolis sampling are shown by a solid line. By using this sampling technique, the only way to make the transition from (0,1) to (1,0) is through one of the energetically unfavorable intermediate states (0,0) or (1,1). The energy barrier associated with this process inhibits accurate sampling of the isoenergetic states (0,1) and (1,0). The modified sampling technique allows transitions between any two states (the additional transitions are shown by dashed lines) thereby improving accuracy in the sampling of the low-energy states.

If the sampling algorithm is trapped in a minimum as described above, the coupled sites that cause the problem are poorly sampled. The resulting average protonations will have larger errors and will be biased toward the minimum in which the trajectory spent most of its time. This bias causes a correlation among the fluctuations in the titration curves of the strongly coupled sites. The correlation and the larger absolute errors make the poorly sampled sites easy to recognize.

We solved this problem by introducing transitions involving *simultaneous* changes of state of these correlated sites; i.e., we included direct proton exchange between the strongly coupled sites in the Monte Carlo scheme. In the illustrative two-site case introduced above, a simultaneous change of the protonation of *both* sites is allowed in addition to the single-site transitions (see dashed line in Fig. 1). This method, which introduces additional transitions in the sampling algorithm, provides an efficient way to sample low-energy states of strongly coupled sites. The probability of transition between any two states in the multisite transition was determined, as in the single-site transition, by the ratio of their Boltzmann factors (i.e., detailed balance was preserved) to ensure that this modified sampling generated the correct equilibrium distribution of protonation states (11).

The effectiveness of this method in reducing the sampling error for two strongly interacting sites is illustrated in Fig. 2. The errors in the protonation values were much larger with the conventional Metropolis algorithm (Fig. 2*a*) than when the sampling was done by the modified algorithm in which a simultaneous change of the protonation of the two residues was allowed (Fig. 2*b*).

Estimation of Errors. Monte Carlo methods are statistical, and their results are subject to sampling error, which must be estimated. The Metropolis algorithm and its modifications guarantee strong correlation between consecutive states; consequently, data are taken only after each complete Monte Carlo step (i.e., after N attempts to change the protonation state, where N is the number of protonatable sites). Nevertheless, correlations between steps persist, and it is necessary to calculate the number of independent "measurements" in the sample. The correlation function (9) for the protonation x (we have dropped the subscript i to simplify

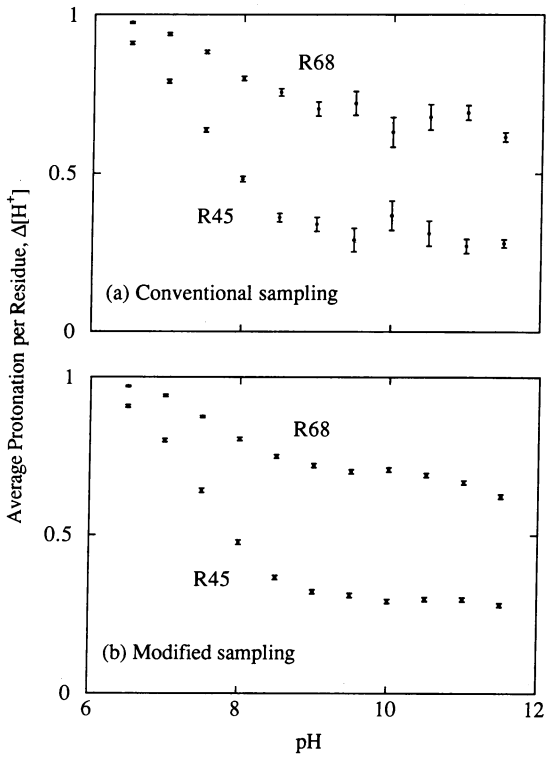

FIG. 2. Protonation vs. pH of the strongly interacting pair of arginines (R45 and R68) in lysozyme. (*a*) Sampling by the conventional Metropolis algorithm; correlation between the protonation of the two sites causes large errors (error bars represent ±1 σ). (*b*) Sampling by a modified algorithm that allows for a simultaneous change in the protonation of the two residues in a single transition (see dashed line in Fig. 1); errors are greatly reduced.

notation) determines the correlation time τ_{corr} between approximately independent measurements and is given by

$$C(\tau) = \frac{1}{T-\tau} \sum_{t=0}^{T-\tau-1} x_{t+\tau}x_t - \langle x \rangle^2, \qquad [5]$$

where t is time in units of Monte Carlo steps, T is total number of steps (or the maximum time), and τ is time variable of the correlation function. The correlation time is the value of τ for which $C(\tau)$ is negligible [we required $|C(\tau)| < C(0)/10$]. The number of independent measurements is T/τ_{corr}.

The variance of one measurement is $C(0)$; use of the average of T/τ_{corr} independent measurements results in the standard deviation

$$\sigma = \left(\frac{C(0)}{T/\tau_{corr}} \right)^{1/2}. \qquad [6]$$

Testing the Monte Carlo Method. To test the accuracy of the Monte Carlo method, we examined a small system, lysozyme, for which exact results can be obtained. If the number of interacting sites is fewer than 25, the 2^N terms in the partition function can be explicitly calculated, and the average protonation of a site is given exactly by Eq. 3. This exact result can be compared with that obtained by the Monte Carlo method.

The intrinsic pK values and site–site interactions used were the same as those used by Bashford and Karplus (4). They assumed that all the arginine residues are protonated throughout the pH range of interest ($0 < pH < 12$); this reduces the number of titrating sites from 32 to 21.

The Monte Carlo results compared well with those from the complete partition function. For 10,000 Monte Carlo steps (i.e., each titratable site sampled on the average of 10,000 times during the Monte Carlo trajectory), the exact value of the protonation for each site fell within one standard deviation of the Monte Carlo value at most values of pH (Fig. 3). The above agreement gives us confidence that the Monte Carlo method accurately represents the protonation states.

Applications

Before the Monte Carlo method is applied, it is necessary to calculate the self energies and site–site interactions that govern the relative probabilities of sampling the different protonation states.

We calculated the electrostatic energies by using a finite difference solution to the linearized Poisson–Boltzmann equation (8) for two systems: one in which the residue is in the protein and the other in which the residue is in solution (4). For each of these systems the electrostatic potential was calculated for the protonated and unprotonated states of the residue. From these values the intrinsic pK values and the site–site interactions were calculated.

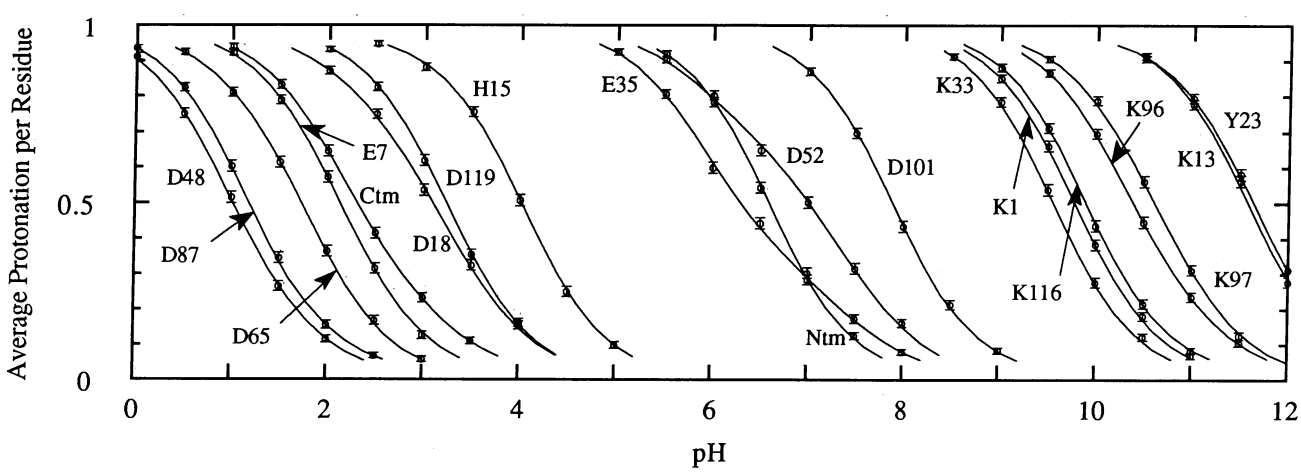

FIG. 3. Titration of the 21 protonatable amino acids (arginine residues not allowed to titrate) in lysozyme (triclinic structure). Smooth curves are the exact calculation of protonation from the partition function (Eq. 3), which agree with the calculation of Bashford and Karplus (4) to within 0.01 proton, and circles are the values calculated from a Monte Carlo simulation of 10,000 steps at intervals of 0.5 pH unit. The exact calculation falls within one standard deviation of the Monte Carlo result for all residues at most pH values. Ctm, C terminus; Ntm, N terminus.

The atomic coordinates used in the calculations were obtained from the crystal structures of the triclinic form of hen egg white lysozyme from the Brookhaven data bank (12) and of the photosynthetic reaction center of *Rb. sphaeroides* (13, 14). Partial charges and atomic radii were assigned to atoms according to the CHARMM parameter set (15). For lysozyme, hydrogen positions were those used in ref. 4; and for the reaction center, hydrogens were added using INSIGHT (a product of Biosym Technologies, San Diego, CA). For titrating sites in the uncharged state, all side-chain atoms had zero charge. The charged state was represented by a point charge on one atom (the carboxyl carbon for aspartic acid, glutamic acid, and the C terminus; the $C_{\epsilon 1}$ atom for histidine; the sulfur atom for cysteine; the hydroxyl oxygen for tyrosine; the guanadino carbon for arginine; and the amino nitrogen for lysine and the N terminus). We assigned a dielectric constant of 4 to the protein interior (16) and of 80 to the solvent. Counterions (10 mM NaCl for lysozyme and 50 mM KCl for the reaction center) were allowed to within 2 Å of the surface (Stern layer). Details of the finite difference technique can be found in ref. 4.

Lysozyme. We calculated the titration of *all* the protonatable residues in lysozyme, including arginines. Inclusion of all the titrating sites in lysozyme provides a simple extension of existing computational results and also tests the previous assumption that the arginines do not contribute throughout the range of titration.

Allowing the arginines to titrate had little effect on other residues titrating in the pH range from 0 to 12 (the maximum change in the pK for the nonarginine residues was 0.2 pK unit), notwithstanding the titration of some arginines below pH 12 (Fig. 4). One interesting feature of the calculation is the behavior of the two arginine residues at positions 45 and 68, whose guanadino carbons are 3.3 Å apart. When the positive charge is localized on the guanadino carbons, the interaction results in a depressed pK value for Arg-45 (see Fig. 2). When the positive charge is distributed more realistically (using the CHARMM partial charges for positively charged arginine rather than a point charge), the coupling is reduced and the order of their titration is reversed.

The measured pK values of the nonarginine residues seem to account for all the protons dissociated in the titration of lysozyme (17, 18); titration of individual arginine residues has not, however, been examined experimentally. It is, therefore, not clear at present whether the one low pK value calculated for the Arg-45,Arg-68 pair is real or the result of simplifying assumptions made in the calculation (see *Discussion*).

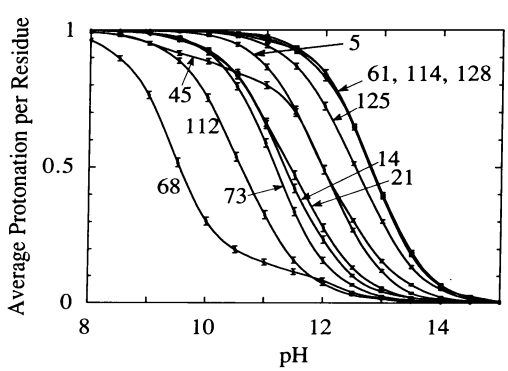

FIG. 4. Titration of the arginine residues in lysozyme. All 32 of the protonatable residues were allowed to titrate (only arginines are shown); the pK values for the nonarginine residues changed by less than 0.2 pK unit. The charge distributions for the protonated states of the arginine residues in this calculation were obtained from the CHARMM partial charge set (15). Use of this more detailed charge distribution (rather than of a point charge) not only reduced the coupling between arginine residues 45 and 68 but also reversed the order in which they titrated (compare with Fig. 2).

Reaction Center. The photosynthetic reaction center of *Rb. sphaeroides*, with 172 titratable amino acids, provides a challenging system for electrostatic calculations. In general, the protonation of a residue is a function of the protonation of all other titrating sites, and we found that considering only pieces of the reaction center gave inconsistent results (the protonation of a residue was a strong function of which residues were allowed to titrate). Since an exact calculation of the 2^{172} terms in the partition function for this system is not feasible, the Monte Carlo technique is well suited for determining the charge distribution within the reaction center as a function of pH.

We examined the proton uptake and free energy change associated with electron transfer between the primary and secondary quinone ($Q_A^- Q_B \rightarrow Q_A Q_B^-$) in the reaction center. The integral of the proton uptake as a function of pH is equal to the change in the free energy (to within a constant of integration) (19). Both the proton uptake and the free energy change have been measured experimentally (19, 20).

Calculation of the net proton uptake by the reaction center during electron transfer requires calculation of the pH dependence of the average protonation of all 172 titratable residues when either Q_A or Q_B is reduced. The calculated proton uptake values $\Delta[H^+](Q_A Q_B \rightarrow Q_A^- Q_B)$ and $\Delta[H^+](Q_A Q_B \rightarrow Q_A Q_B^-)$ were compared directly with experimental values, and the integral of $\Delta[H^+](Q_A^- Q_B \rightarrow Q_A Q_B^-)$ was compared with the measured free energy $\Delta G(Q_A^- Q_B \rightarrow Q_A Q_B^-)$.

For the reduced quinones the negative charge was placed at the center of the quinone ring. More realistic charge distributions were found to have little effect on the protonation of the residues. The presence of detergent was not included in the calculation.

The calculated free energy curve $\Delta G(Q_A^- Q_B \rightarrow Q_A Q_B^-)$ as a function of pH is in fair agreement with the experimentally determined curve (Fig. 5). However, the individual proton uptake values $\Delta[H^+](Q_A Q_B \rightarrow Q_A^- Q_B)$ and $\Delta[H^+](Q_A Q_B \rightarrow Q_A Q_B^-)$ agree with the experimental values only within a factor of two.

Discussion

We applied a Monte Carlo method to calculate the protonation of many interacting sites in a protein. The method was tested and validated on a small test system (lysozyme) for which the complete partition function was calculated. We

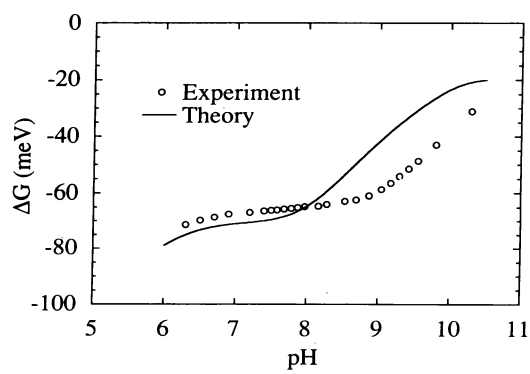

FIG. 5. Free energy change associated with electron transfer from Q_A to Q_B in the photosynthetic reaction center of *Rb. sphaeroides*. Circles are experimental data (20). The theoretical curve was obtained by integrating the differences in the calculated proton uptakes $\Delta[H^+](Q_A Q_B \rightarrow Q_A^- Q_B)$ and $\Delta[H^+](Q_A Q_B \rightarrow Q_A Q_B^-)$. The constant of integration was chosen to give agreement with the experimental value at pH 8.

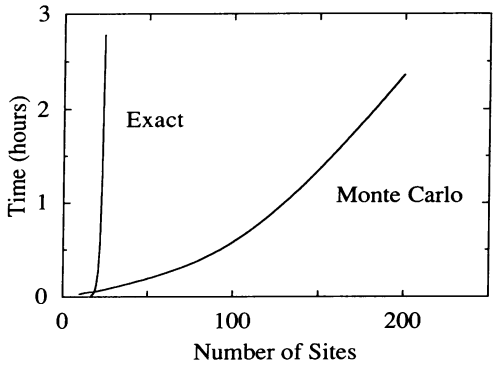

FIG. 6. Time required to determine the average protonation of N residues (at one value of pH) by an exact calculation and by Monte Carlo sampling (10,000 Monte Carlo steps). Calculations were performed on a Solbourne 5-502 (3.4 megaFlops). Beyond ≈25 sites the time required to compute the exact solution becomes prohibitive, whereas the Monte Carlo calculation remains practical for several hundred sites.

found that about 10,000 Monte Carlo steps gave average protonation values with an absolute error of ≈0.02 proton. Even for proteins as large as the reaction center (172 sites), the time required for sampling is reasonable, whereas the time required to calculate the average protonation exactly becomes prohibitive for more than ≈25 sites (Fig. 6). Thus, the calculation of the protonation of the 172 interacting residues in the reaction center could only be accomplished by the Monte Carlo method.

We found qualitative agreement between the calculated and observed protonation of the reaction center when Q_A and Q_B were reduced. We attribute the quantitative discrepancies between theory and experiment to inaccuracies in the calculation of the electrostatic energies (i.e., to the input to the Monte Carlo sampling rather than to the Monte Carlo method).

Now that we have an adequate procedure to treat the statistical problem of many titrating sites, the calculation of electrostatic energies needs to be refined. Possible improvements in these calculations involve the following points: (*i*) inclusion of bound-detergent molecules and interior water molecules [water positions were not reported for *Rb. sphaeroides* but were observed in the structure of *Rhodopseudomonas viridis* (21)], (*ii*) allowing the protein to relax in response to electric fields, (*iii*) more realistic charge distributions for the titrating sites, (*iv*) a more sophisticated treatment of counterions, especially those that bind to the protein, and (*v*) a more realistic treatment of the dielectric properties of the interior of the protein.

In addition, we have assumed that all protonatable residues can exchange protons with and are in thermal equilibrium with the external solution. If no mechanism for protonation of a residue exists or if the time scale for proton exchange is longer than the time scale of the experimental measurements, the calculation needs to be modified.

In conclusion, we have shown that the Monte Carlo method is well suited to solve the statistical part of the protonation problem of many interacting residues. To obtain better agreement between theory and experiment, the calculation of the electrostatic self energies and interaction energies needs to be improved.

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