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Acidic-Basic Properties of Three Alanine-Based Peptides Containing Acidic and Basic Side Chains: Comparison Between Theory and Experiment

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Abstract

The purpose of this work was to evaluate the effect of the nature of the ionizable end groups, and the solvent, on their acid-base properties in alanine-based peptides. Hence, the acid-base properties of three alanine-based peptides: Ac-KK-(A)₇-KK-NH₂ (KAK), Ac-OO-(A)₇-DD-NH₂ (OAD), Ac-KK-(A)₇-EE-NH₂ (KAE), where A, D, E, K, and O denote alanine, aspartic acid, glutamic acid, lysine, and ornithine, respectively, were determined in water and in methanol by potentiometry. With the availability of these data, the ability of two theoretical methods to simulate pH-metric titration of those peptides was assessed: (i) the electrostatically driven Monte Carlo method with the ECEPP/3 force field and the Poisson-Boltzmann approach to compute solvation energy (EDMC/PB/pH), and (ii) the molecular dynamics method with the AMBER force field and the Generalized Born model (MD/GB/pH). For OAD and KAE, pK_{a1} and pK_{a2} correspond to the acidic side chains. For all three compounds in both solvents, the pK_{a1} value is remarkably lower than the pK_a of a compound modeling the respective isolated side chain, which can be explained by the influence of the electrostatic field from positively charged ornithine or lysine side chains. The experimental titration curves are reproduced well by the MD/GB/pH approach, the agreement being better if restraints derived from NMR measurements are incorporated in the conformational search. Poorer agreement is achieved by the EDMC/PB/pH method.

Keywords

protonation state; potentiometric titration; pH-dependent conformation; pH-calculations; Poisson-Boltzmann equation; Generalized Born model

INTRODUCTION

Detailed knowledge of the pH-dependence of the proton-dissociation constants of acidic and basic groups in both folded and unfolded states of proteins is necessary for an understanding of the role of electrostatics in protein stability.¹ The protonation state of a titratable group is determined by the solvent pH and the pK_a of the group. The pK_a of a given group, in turn, is influenced by its electrostatic environment, which is determined by the protein conformation and protonation state of other titratable groups.² However, predicting how the solvent modulates conformational transitions and influences pK_a values that regulate the biological

functions of biomolecules remains an unsolved challenge,³ although many algorithms have been developed to treat this problem.^{2–12}

In this work, we have evaluated the effect of the nature of the ionizable end groups, and the solvent, on their acid-base properties in three alanine-based peptides. To achieve this goal, we have measured the potentiometric titration curves of the following three alanine-based peptides in water and methanol: Ac-KK-(A)₇-KK-NH₂ (KAK), Ac-OO-(A)₇-DD-NH₂ (OAD), Ac-KK-(A)₇-EE-NH₂ (KAE), where A, D, E, K, and O denote alanine, aspartic acid, glutamic acid, lysine, and ornithine residues, respectively. Having these titration curves available, we assessed the ability of two different approaches to calculate these titration curves, namely (i) the electrostatically driven Monte Carlo method¹³ with the ECEPP/3 force field¹⁴ and the Poisson-Boltzmann approach to calculate the solvation free energy (EDMC/PB/pH),⁸ and (ii) the molecular dynamics method with the AMBER force field¹⁵ and the Generalized Born¹⁶ model (MD/GB/pH),¹² respectively. The purpose of the theoretical calculations was to determine how state-of-the-art methods of constant-pH molecular simulations perform in treating these titration data.

We previously carried out similar studies^{7,17,18}; in particular, in recent work,¹⁸ we studied the sequence Ac-XX-(A)₇-NHMe (XAO), where X denotes the diaminobutyric acid residue. This peptide has received a lot of attention because it was considered to be a model of polyproline conformation.^{19–22} We, therefore, considered it worthwhile to investigate the influence of the length and kind of the ionizable side chain on its acidic-basic properties in the alanine-based peptide compared to that in the isolated state, for which an appropriate model compound was chosen for each of them (*n*-butylamine for lysine, *n*-propylamine for ornithine, acetic acid for aspartic acid, and propionic acid for glutamic acid, respectively). In particular, OAD and KAE contain acidic and basic groups on both ends, which could lead to formation of salt bridges between ionized carboxylate groups and protonated amino groups, thereby decreasing the pK_a values of the carboxyl groups, substantially, as found in our earlier work.¹⁸ In an accompanying paper,²³ we report a conformational study of KAK, OAD, and KAE by CD and NMR spectroscopy and theoretical methods to assess the effect of these ionizable groups on conformation.

RESULTS AND DISCUSSION

Figure 1 presents the experimental and fitted titration curves of the peptides in water and in methanol, respectively. It can be seen that the model of four stepwise proton-dissociation equilibria fits the experimental points very well for the three peptides in both solvents. The pK_a values calculated from the titration data by using the program STOICHIO^{24,25} are collected in Table I.

For OAD and KAE, the pK_{a1} and the pK_{a2} values are remarkably lower than pK_{a3} and pK_{a4} (Table I) and, consequently, must correspond to the side chains of the acidic residues. It should be noted, however, that the pK_{a1} value is always smaller than that of the corresponding model compound (Table II; Refs. 18^{26–30}) (pK_{a1} = 3.46, pK_{a2} = 4.67 for OAD in water, pK_{a1} = 6.35, pK_{a2} = 6.91 for OAD in methanol, pK_{a1} = 4.24, pK_{a2} = 5.22 for KAE in water, and pK_{a1} = 5.81, pK_{a2} = 6.66 for KAE in methanol). The lowering of the pK_a value of the first carboxyl group can be explained by the influence of the electrostatic field of the positively charged protonated amino groups at the other end of a molecule. The pK_{a2} value is either smaller or greater than that of a model compound, because of the competing effect of the negative charge of the already deprotonated carboxyl group and the positive charge of protonated lysine or ornithine side chains. This observation is in agreement with the fact that, in water, the first two acidity constants are more differentiated for OAD than for KAE, because the first peptide has shorter side chains and, consequently, the groups are more influenced by the electrostatic field of the

other side chains. For the KAK peptide all four values of pK_a are also less than or nearly equal to those of the model compound, which can be explained by the influence of the positive charge of the other, not yet deprotonated ammonium group. The difference in the pK_a values is much less pronounced in KAK than in the XAO peptide,¹⁸ for which the consecutive pK_a values are 2.72, 8.78, 10.17, 11.06 in water and 5.44, 9.90, 11.51, 13.37 in methanol, respectively. As for OAD and KAE, this observation can be explained by the greater length of the lysine side chain compared to that of ornithine or diaminobutyric acid and, consequently, much smaller influence of the electrostatic field of the other charged group on the apparent proton affinity of the group under consideration.

The titration curves calculated for all three peptides studied with the EDMC/PB/pH and the MD/GB/pH approaches in water and methanol are shown in Figure 2. For the MD/GB/pH approach, we used both the regular procedure in which a free MD run is carried out with possible protonation states sampled after every given number of steps and we also used the set of conformations generated by MD runs with time-averaged restraints from NMR measurements²³; it should be noted that the restraints were obtained from NMR measurements at pH = 5.7.

It can be seen (see Figure 2) that, as in our previous study,¹⁸ for all three peptides both in water and in methanol, better agreement between theory and experiment is obtained for the MD/GB/pH method compared to that of the EDMC/PB/pH method; the agreement is improved further if conformations obtained from MD runs with experimental restraints are used. The low pH values at the beginning of the titration of KAK apparently result from an excess of acid (most probably trifluoroacetic acid used in the purification procedure; “Peptide Synthesis” section). This excess was estimated by fitting the equilibrium model to the experimental points with STOICHIO to be about 0.4 mol fraction. We also considered the possibility of excess acid in OAD and KAE; however, the value estimated by fitting was effectively 0.

The degrees of protonation of the titratable groups of all three peptides, calculated with the use of the MD/GB/pH approach in water and in methanol, are shown in Figure 3. From the protonation curves calculated by the MD/GB/pH approach, we have determined the theoretical pK_a values of the ionizable groups of the peptides studied as the pH values at which the average degree of deprotonation of a given group is 0.5. By arranging the theoretically calculated pK_a values in the order of those determined by the potentiometric titrations, we approximately assigned the experimentally determined pK_a s to the ionizable groups present in the polypeptides studied; this information is also shown in Table I. It can be seen from Table I that the differences between the pK_a values, determined potentiometrically and those calculated, range from 0.01 to 1.3, for water and from 0.02 to 2.5 for methanol, respectively. The mean values and the standard deviations of the errors in pK_a are 0.5 and 0.4 for water and 0.9 and 0.8 for methanol, respectively. The greater error of theoretically calculated pK_a s in methanol suggests that the GB model is better tuned for water than for nonaqueous solutions; however, the experimental pK_a s determined in nonaqueous solutions (both those of the model compounds collected in Table II and those of the peptides studied) involve greater error compared to those determined in water. It can also be noted that the calculated pK_a values of the side chains of the same kind span a narrower range than those determined potentiometrically.

It can be seen (see Figure 3) that, for OAD and KAE, the carboxyl groups deprotonate first, the second one losing protons at a higher pH than the first one, which is in agreement with the pK_a values determined from the potentiometric measurements (Table I). For KAK the second proton is dissociated from a lysine side chain (K2 for water or K1 for methanol) located at the same end of the molecule as that already deprotonated (K1 for water or K2 for methanol). We should note that, because the differences between the pK_a values determined by potentiometric

titration and calculated by the MD/GB/pH method are of the same order as the differences between the pK_a values of the consecutive lysine side chains, the order of deprotonation of the lysine side chains stated above should be treated as approximate.

CONCLUSIONS

By using the potentiometric-titration method and subsequent titration-curve fitting, we determined the pK_a values of three alanine-based peptides differing by the kind and length of the side chains of the ionizable amino acid residues. The variation of the pK_a values of the compounds studied can be explained qualitatively by the influence of the electrostatic field from one group on the other group being deprotonated: for OAD and KAE, the value of pK_{a1} is lower than that of a model compound representing a given side chain (see Tables I and II) because of the influence of the positive electrostatic field of the protonated basic residues from the other end of the molecule, while pK_{a2} is either greater or smaller because of the influence of the electric field from the negatively charged deprotonated neighboring carboxylate group and that of the positively charged ornithine or lysine side chains. For KAK, pK_{a1} (in water) or pK_{a1} and pK_{a2} (in methanol) are by about 1 unit lower than pK_{a2} , pK_{a3} , and pK_{a4} (in water) or pK_{a3} and pK_{a4} (in methanol) (see Tables I and II) because of the influence of the positive charge from a neighboring positively charged protonated amino group.

We compared the potentiometric titration curves of the KAK, OAD, and KAE peptides calculated by using two theoretical methods, EDMC/PB/pH and MD/GB/pH, respectively. As in our earlier work, the second approach gives better agreement with experiment which supports the conclusion of our previous study¹⁸ that the AMBER force field with the GB solvation model reflects the energetics of protonation in water better than ECEPP/3 with the PB solvation model. The best agreement was obtained when conformations generated by MD with time-averaged restraints from NMR measurements were used to compute average protonation states at different pH; this demonstrates that the set of conformations used to calculate the protonation states is important. In fact, the conformations obtained in unrestrained MD runs with AMBER plus the GB solvation models had well-developed α -helical segments contrary to experiment²³; therefore, imposing restraints from NMR measurements is necessary to obtain the conformational ensemble of good quality.

MATERIALS AND METHODS

Experimental

Chemicals—All Fmoc amino acids were purchased from Peptides International. 1-Hydroxybenzotriazole (1-HOBt), piperidine and trifluoroacetic acid (TFA) were purchased from Lancaster. 1,3-Diisopropylcarbodiimide (DIPCI), *N*-acetylimidazole, triisopropylsilane, phenol, and potassium hydroxide were obtained from Sigma-Aldrich. Acetonitrile for high-performance liquid chromatography (HPLC) was obtained from LabScan. Methanol (HPLC grade) was purchased from Sigma-Aldrich and contained less than 0.02% of water. All compounds were used without further purification. Water was purified using the Milli-Q system (Millipore) whereas *N,N*-dimethylformamide (DMF; obtained from LabScan) was distilled under reduced pressure and then kept over a 0.4-nm molecular sieve.

Peptide Synthesis—The peptides were synthesized on a 0.19 mmol scale by the solid-phase method using the Fmoc strategy with an automated 9050 Plus PepSynthesizer (Millipore). *N*^α-Fmoc amino acids were in a fourfold excess over the required amount of each peptide, and DIPCI was used as a coupling agent. The syntheses were carried out in the presence of 1-HOBt. The Fmoc group was removed using 20% piperidine in DMF added to the resin-bound peptide, followed by acetylation with 10 cm³ of 1M *N*-acetylimidazole solution in DMF for about 18 h at room temperature. Cleavage of the peptides from the resin was performed with 10 cm³ of

TFA, triisopropylsilane, water, and phenol (88:2:5:5) for 2 h at room temperature. The resin was removed by filtration and washed three times with TFA (each with 10 cm³ of TFA). After evaporation of the solvent in vacuo, the resulting oily residues were washed twice with diethyl ether (with that operation repeated three times), and then lyophilized. Purification was carried out on a Kromasil-5-C8 (20 mm × 250 mm) preparative HPLC column applying a linear gradient of 10–30% 80% acetonitrile/water/0.1% TFA with a flow rate of 12 cm³/min for KAK, a linear gradient of 0–20% 80% aceto-nitrile/water/0.1% TFA with a flow rate of 12 cm³/min for OAD, and a linear gradient of 5–25% 80% acetonitrile/water/0.1% TFA with a flow rate of 12 cm³/min for KAE. The purity of the peptides was confirmed by analytical HPLC and MALDI-TOF analysis.

Potentiometric Titration—All measurements were carried out at (298.1 ± 0.1) K in an EMF cell containing a glass electrode and a calomel electrode. The potentiometric microtitration unit was equipped with a computer-aided microtitrator system and a millivoltmeter with accuracy of 0.01 mV. An ESH 10-00 (METRON) glass microelectrode was used as the indicator electrode, and a calomel EK 10-00 microelectrode, modified by replacing the internal saturated KCl solution with a 0.1M tetra-*n*-butylammonium chloride solution in the appropriate solvent (water or methanol), was used as the reference electrode. The titrant was added dropwise using a 2.5-cm³ HAMILTON syringe with an accuracy of 0.01 cm³.

For each titration, a stock solution of the peptide under study was prepared at a concentration of ~0.001M; 3 cm³ of this solution was added to the titration vessel. The solutions of the peptides in water were titrated with 0.01M potassium hydroxide in water, while the solutions of the peptides in methanol were titrated with 0.01M tetrabutylammonium hydroxide in methanol except for OAD and KAE in methanol, where the base concentration of the titrant was 0.015 and 0.02M, respectively. Before running each series of measurements, the electrode was calibrated as described in Ref. 17.

For water, the EMF of five standard buffer solutions from Merck with pH = 2, 4, 7, 9, and 12, respectively, was measured, and the Nernst equation $E = E^0 - S \times \text{pH}$, where E^0 and S are the intercept and the slope, respectively, was fitted to the measured data. A linear fit was obtained with $S = 58.36 \pm 0.77$ mV/pH unit; $E^0 = 387.93 \pm 5.82$ mV. For methanol, the electrode was calibrated against the 2,6-dinitrophenol/tetra-*n*-butylammonium 2,6-dinitrophenolate system with a $\text{p}K_{\text{a}}^{\text{MeOH}}(2,6\text{-NO}_2\text{PhOH}) = 7.8$.¹⁴ A linear fit was obtained with $S = 60.91 \pm 0.98$ mV/pH unit; $E^0 = 567.80 \pm 8.4$ mV.

Theoretical Considerations

Fitting Models of Chemical Equilibria to Titration Curves—Models of acid-base equilibria were fitted to the resulting titration curves by using the program STOICHIO,^{24,25} which is based on a nonlinear confluence analysis. This program can treat any model of chemical equilibria and takes into account all possible sources of experimental error (the error in EMF, titrant and titrated solution volume, reagent impurities, etc.; see above references for details of the determination of the $\text{p}K_{\text{a}}\text{s}$). The standard deviations of the titrant volume and of the EMF were assumed to be $\sigma_{\text{V}} = 0.001$ cm³ and 1.0 mV, respectively. All peptides were treated as tetrafunctional acids, and all four $\text{p}K_{\text{a}}$ values were considered as parameters to be determined. The concentration of water in methanol was assumed to be 10⁻²M with a standard deviation of 10⁻²M. The model of chemical equilibria consisted of stepwise dissociation of the protons from the peptide and acidic dissociation of water which was introduced to the system by titration of the methanol solution with tetra-*n*-butylammonium hydroxide, which produces water upon reaction with acidic groups of the peptide. We also took into account the fact that the peptides could contain excess of acid (most probably a residual amount of TFA) as a result of the purification procedure (see “Peptide Synthesis” section) and, consequently, adjusted the

amount of excess acid assuming a standard deviation of this quantity of 0.05 mol fraction of the peptide.

Theoretical Calculations—We used two approaches (EDMC/PB/pH and MD/GB/pH) for the calculations. The EDMC/PB/pH method⁸ consists of two stages. First, a conformational search with the EDMC method¹³ and the ECEPP/3¹⁴ force field with the SRFOPT³¹ solvent-accessible surface area model is carried out. Subsequently, the energies of the conformations generated in this way are re-evaluated by using a more accurate energy function, $E(\mathbf{r}_p, \text{pH})$, defined by Eq. (1) at all pH values considered.

$$E(\mathbf{r}_p, \text{pH}) = E_{\text{int}}(\mathbf{r}_p) + \Delta F_{\text{cav}}(\mathbf{r}_p) + F_{\text{solv}}(\mathbf{r}_p) + F_{\text{inz}}(\mathbf{r}_p, \text{pH}) \quad (1)$$

where $\mathbf{r}_p$ denotes the coordinates of the p th conformation, $E_{\text{int}}(\mathbf{r}_p)$ is the internal conformational energy of the molecule in the absence of solvent, assumed to correspond to the ECEPP/3 energy of the neutral molecule, $F_{\text{cav}}(\mathbf{r}_p)$ is the free energy of cavity creation, $F_{\text{solv}}(\mathbf{r}_p)$ is the free energy of polarization of the solvent, and $F_{\text{inz}}(\mathbf{r}_p, \text{pH})$ is the free energy of the change in the state of ionization of the ionizable groups due to the transfer of the molecule from the gasphase to the solvent, at a fixed pH value. The EDMC calculations were started from randomly generated structures. A detailed description of these energy terms can be found in our earlier work.⁸ All EDMC calculations were carried out with the ECEPPAK package.^{32,33} A total of about 1500 energy-minimized and accepted conformations were collected and their energies re-evaluated using Eq. (1).

The MD/GB/pH method¹² is based on the GB solvation model, with periodic Monte Carlo sampling of the protonation states. At each Monte Carlo step, a titratable site and a new protonation state for that site are chosen randomly. A change in free energy, ΔG , for the protonation or deprotonation is calculated according to Eq. (2).¹²

$$\Delta G = k_B T (\text{pH} - \text{p}K_{a,\text{ref}}) \ln 10 + \Delta G_{\text{elec}} - \Delta G_{\text{elec,ref}} \quad (2)$$

where k_B is the Boltzmann constant, T is the absolute temperature, pH is the specified solvent pH, $\text{p}K_{a,\text{ref}}$ is the $\text{p}K_a$ of the appropriate reference compound, ΔG_{elec} is the electrostatic component of the change in free energy calculated for the titratable group in the protein, and $\Delta G_{\text{elec,ref}}$ is the electrostatic component of the change in free energy for the reference compound. More details of the implementation of the approach can be found in our previous work.¹⁸

Molecular dynamics calculations were carried out using the AMBER 8 suite of programs¹⁵ with the ff99 force field in the NTV ensemble at temperature $T = 300$ K. The integration step was 1 fs. The simulations were run at successive increments of one unit of pH from 2 to 14 for water, and from 4 to 15 for methanol. The range of pH was chosen based on the value of the ionic product of each solvent. 1,000,000 steps were run for each pH value, and snapshots were collected every 250 steps (a total of 400,000 snapshots for every pH value). The last 200,000 snapshots were taken to compute the titration and degree of protonation curves (Figures 2 and 3). In computing the electrostatic-solvation energy with the GBSA model (16), the ionic strength was assumed to be 0.1M. The convergence of sampling was checked by comparing the calculated plots of the degree of ionization of each titratable group vs. pH corresponding to different slices of the trajectories; we found that the curves were overlapping after 200,000 snapshots for each compound.

To evaluate the F_{cav} and F_{solv} components in Eq. (1) and to estimate ΔG_{elec} in Eq. (2), the surface tension and the dielectric constant of water and methanol are required. These constants are summarized in Table III.^{34–36} As reference compounds for the side chains, we used *n*-butyl amine (*n*-BuNH₂) for lysine, acetic acid for aspartic acid, propyl amine for ornithine, and propionic acid for glutamic acid, respectively. We consider these model compounds more appropriate than terminally blocked amino acid residues as references, because they contain only the immediate hydrocarbon surrounding of the respective ionizable groups without possible interactions with the polar backbone part, which should be handled within the framework of the Poisson-Boltzmann or Generalized Born model. Their pK_a values in water and methanol are collected in Table II (Refs. 18, 26–30).

In the MD/GB/pH approach, canonical sampling of both conformation and protonation states is carried out. Therefore, the average number of protons attached to the peptide molecule at a given pH is computed as a direct average over conformations. In the EDMC/PB/pH approach, which involves noncanonical sampling, the average number of protons of the peptide under study is calculated by Boltzmann averaging over the energies of the conformations of the ensemble.¹⁷

To compare the theoretical titration curves with the corresponding experimental titration curves, we converted, $\langle x(\text{pH}) \rangle$ [the charge of the polypeptide under consideration averaged over conformations; see Eqs. (2–4) in Ref. 17] into volume of added titrant [$V(\text{pH})$] as given by Eq. (3) for KAK and Eq. (4) for KAE and OAD, respectively.

$$V(\text{pH}) = \frac{\{(4 - \langle x(\text{pH}) \rangle)C_p - 10^{-\text{pH}} + C_w 10^{(\text{pH} - pK_{aw})}\}}{C_{OH} + 10^{-\text{pH}} - C_w 10^{(\text{pH} - pK_{aw})}} V_p \quad (3)$$

$$V(\text{pH}) = \frac{\{(2 - \langle x(\text{pH}) \rangle)C_p - 10^{-\text{pH}} + C_w 10^{(\text{pH} - pK_{aw})}\}}{C_{OH} + 10^{-\text{pH}} - C_w 10^{(\text{pH} - pK_{aw})}} V_p \quad (4)$$

where C_p is the concentration of the peptide in the titrated solution, V_p is the volume of the titrated solution, C_{OH} is the concentration of the titrant, C_w is the concentration of water in the solution for nonaqueous solvents, and K_{aw} is the acid-dissociation constant of water in a given solvent. For nonaqueous solvents, traces of water must be taken into account because traces of water of the order of $10^{-3}M$ can act as a base or an acid even comparable to the concentration of reagents in potentiometric titrations.^{17,18} The factors of 4 and 2 in Eqs. (3) and (4), respectively, appear because all side chains are protonated at the beginning of a titration; therefore, the charge is 4 for KAK (four basic side chains) and 2 for OAD and KAE (two basic side chains), while at the point of deprotonation of all titratable groups the charge is 0 for KAK and -2 for OAD and KAE, respectively).

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FIGURE 1.

Potentiometric-titration curves of KAK (a and d), OAD (b and e), and KAE (c and f) in water (a–c) and methanol (d–f). Circles: experimental points, solid lines: fit of the experimental data to the model of four consecutive proton-dissociation equilibria (“Fitting Models of Chemical Equilibria to Titration Curves” section). The vertical lines are drawn at volumes corresponding to full neutralization of the subsequent protonated groups.

FIGURE 2.

Comparison of experimentally determined (solid lines) and calculated titration curves of KAK (a and d), OAD (b and e), KAE (c and f) in water (a–c) and methanol (d–f). Long dashed lines: the EDMC/PB/pH approach; short dashed lines: the MD/GB/pH approach with free MD run; dotted lines: the MD/GB/pH approach with the set of conformations generated by using MD calculations with the NMR restraints (water only).

FIGURE 3.

Plots of the computed pH vs. the degree of protonation ($\langle x_i \rangle$) of the four titratable groups of the KAK (a and d), OAD (b and e), and KAE (c and f) peptides obtained by using the MD/GB/pH method in water (a–c) and in methanol (d–f). Solid lines: K_1 (KAE and KAK) or O_1 (OAD); short dashed lines K_2 (KAE and KAK) or O_2 (OAD); dotted lines E_{10} (KAE), K_{10} (KAK), or D_{10} (OAD); long-dashed lines E_{11} (KAE), K_{11} (KAK), or D_{11} (OAD). The number following the one-letter symbol of an amino acid residue indicates its position in the sequence. The pK_a values of the ionizable groups calculated from the plots are given in Table I.

Table 1
The pK_a Values of KAK, OAD, and KAE, and their Standard Deviations, in Water and Methanol at 298 K^a

	KAK		OAD		KAE	
	H ₂ O	MeOH	H ₂ O	MeOH	H ₂ O	MeOH
pK_{a1}	9.10 (0.15)	7.95 (0.12)	3.46 (0.16)	6.35 (0.22)	4.24 (0.10)	5.81 (0.14)
	9.80 (K1)	8.78 (K2)	3.10 (D11)	8.57 (D10)	4.3 (E10)	5.45 (E11)
pK_{a2}	10.16 (0.51)	8.37 (0.20)	4.67 (0.07)	6.91 (0.16)	5.22 (0.06)	6.66 (0.11)
	9.89 (K2)	8.89 (K1)	3.39 (D10)	8.61 (D11)	4.49 (E11)	5.87 (E10)
pK_{a3}	10.25 (0.51)	9.49 (0.19)	9.85 (0.05)	10.23 (0.10)	9.91 (0.04)	8.40 (0.09)
	10.01 (K10)	9.45 (K10)	9.59 (O2)	10.25 (O2)	9.9 (K1)	10.94 (K2)
pK_{a4}	10.6 (1.1)	10.22 (0.27)	10.99 (0.19)	11.99 (0.08)	11.29 (0.22)	11.70 (0.07)
	10.16 (K11)	10.27 (K11)	10.21 (O1)	10.99 (O1)	10.12 (K2)	10.99 (K1)

^aUpper rows and regular font: values determined by fitting the model of four stepwise proton-dissociation protonation equilibria (“Fitting Models of Chemical Equilibria to Titration Curves” section) to the potentiometric-titration curves with standard deviations in parentheses. Lower rows and italics: values calculated from the results of MD/GB/pH simulations (“Theoretical Calculations” section) as pH values corresponding to the average degree of protonation of a given group equal to 0.5 (the one-letter symbols of the corresponding amino acids and their positions in the sequence are in parentheses).

Table IIThe pK_a Values of Reference Model Compounds at 298 K Used in the Calculations

Model	Amino Acid	pK_a in H ₂ O	pK_a in MeOH
<i>n</i> -Butyl amine	Lysine	10.50	11.22 ^a
Propyl amine	Ornithine	10.71 ^b	12.65 ^c
Propionic acid	Glutamic acid	4.87 ^d	7.1 ^e
Acetic acid	Aspartic acid	4.76 ^f	9.67 ^f

^aRef. 28.^bRef. 29.^cRef. 18.^dRef. 27.^eRef. 26.^fRef. 30.

Table III

Physicochemical Constants of Solvents at 298 K Used in the Calculations of Physicochemical Quantities

Solvent	Abbreviation	σ^a (dyne/cm)	ϵ^b
Water	H ₂ O	72.0 ^c	80.0 ^d
Methanol	MeOH	22.6 ^e	32.6 ^d

^aSurface tension.^bDielectric constant.^cRef. 34.^dRef. 35.^eRef. 36.