An NMR and ab Initio Quantum Chemical Study of Acid−Base Equilibria for Conformationally Constrained Acidic α-Amino Acids in Aqueous Solution

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Abstract: The protonation states of a series of piperidinedicarboxylic acids (PDAs), which are conformationally constrained acidic α-amino acids, have been studied by ^{13}C NMR titration in water. The resulting data have been correlated with theoretical results obtained by HF/6-31+G* calculations using the polarizable continuum model (PCM) for the description of water. The PDAs are highly ionizable and contain one or two possible internal hydrogen bonds. In the present study, we show that the PCM model is able to reproduce the relative stabilities of the different protonation states of the PDAs. Furthermore, our results show that prediction of relative pK_a values for two different types of ionizable functional groups covering a pK_a range from 1.6 to 12.1 is possible with a high degree of accuracy.

Introduction

The acidic α-amino acids glutamic acid and aspartic acid (Figure 1) are the major excitatory neurotransmitters in the central nervous system.[1] Imbalance of excitatory neurotransmission seems to be implicated in a number of neurological disorders such as Alzheimer's disease, Huntington's chorea, and epilepsy.[2] Therefore, design and synthesis of novel potent and specific ligands for excitatory amino acid receptors, with the aim to develop therapeutics that are able to prevent or cure these diseases, is currently a very active area of research.[2] The recently reported high-resolution crystal structure of the binding domain of a glutamic acid receptor (GluR2)[3] constitutes a breakthrough, making structure-based design of novel ligands feasible.

In connection with computer-aided ligand design and analysis of ligand−receptor interactions, it is necessary to be able to compute a number of physicochemical properties of potential ligands. These include conformational energies and populations of protonation states in aqueous solution. A major problem in this context is the very high polarity and ionizability of ligands of excitatory amino acid receptors. The computation of physicochemical properties for such molecules is very challenging due to large solvation effects on the charged species and the possibility of formation of very strong internal hydrogen bonds.

We have previously studied various aspects of the interaction between the carboxylate group and the ammonium ion, such as the performance of ab initio continuum models in predicting the equilibrium between the ion-pair and neutral complexes of trimethylamine and formic acid,[4] the interaction of ammonium carboxylate with an aromatic moiety,[5] and the conformational preference of the β-alanine zwitterion in water.[6] During this work, it has become apparent that one of the factors limiting theoretical development and applications within this area is the lack of accurate experimental data regarding structures and energies of highly polar compounds in aqueous solution. We have therefore undertaken a combined experimental and theoretical study of selected model systems. Acidic α-amino acids such as glutamate and aspartate are conformationally flexible molecules, making a correlation between calculations and experimental results complex and uncertain. In search for simple, conformationally constrained analogues, we noted that the piperidinedicarboxylic acids (PDAs) fulfill our criteria. These compounds are expected to be triply ionized in neutral aqueous solution. Due to the presence of the relatively rigid six-membered ring, only a few conformations need to be determined for each species in order to cover the relevant conformational space. On the other hand, various positions and relative configurations of the carboxylic groups in the piperidine ring of PDAs allow studies of the behavior of the functional groups under different but related conditions.

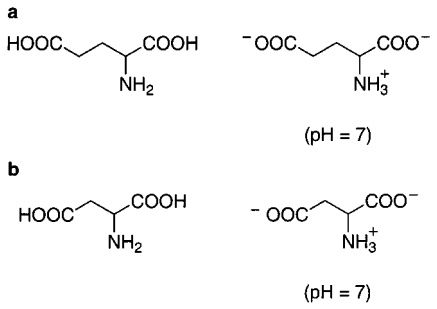

Figure 1. (a) Glutamic acid and (b) aspartic acid in neutral and fully ionized forms.

In the present study, the results of experimental and computational studies of ionization equilibria (pK_a values) for six PDAs are reported. The compounds studied are shown in Figure 2. Compounds **1** and **2** are conformationally constrained analogues of aspartic acid, whereas compounds **3** and **4** and **5** and **6** are

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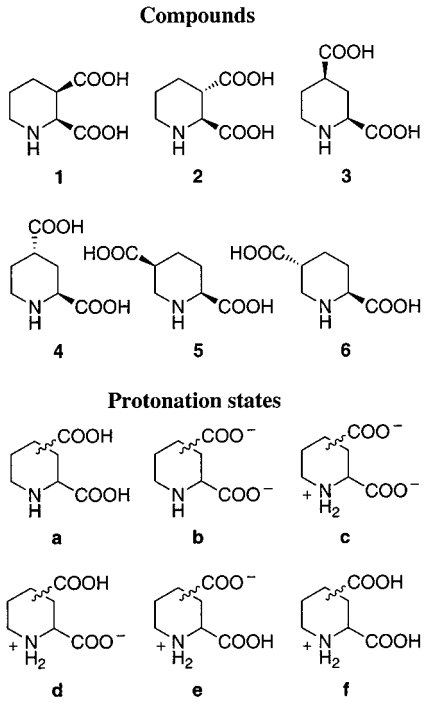

Figure 2. Piperidine dicarboxylic acids (PDAs) studied in the present work. Lower case letters designate the protonation states.

cyclic analogues of glutamic acid and α-aminoadipic acid, respectively.

Prediction of pK_a values has long been a challenge to theoretical chemists. Several approaches have been advanced, ranging from use of empirically determined functional group contributions to high-level quantum-mechanical calculations. The profound influence of solvent on pK_a values is well recognized. Thus, the rank order of gas phase acidities of a series of compounds may be substantially different from that observed in solution. Acidity models can be based on the electrostatic properties of either the acid or the conjugated base, but such treatments cannot fully represent the true equilibrium situation. For isodesmic cases, a good agreement between experimentally determined and predicted results may be found at relatively low levels of theory. For more complex predictions, it is clear that both the underlying level of theory and the solvation model must be accurate, and moreover, they must work well together. In general, most studies show that predictions of absolute pK_a values yield large errors. The failure in prediction of absolute pK_a values has been shown in part to be due to inaccurate estimations of free energies of deprotonation in the gas phase and, in part, to strong interactions between charged species and the solvent.[7−10] Almost all successful implementations involve calibration to a relative pK_a scale using experimentally determined values.[9,11] Relative pK_a values for series of related compounds have been shown to be predictable, but problems arise when compounds belonging to different classes are compared.[7,9,10,12]

Methods and Materials

Chemicals. *cis*-2,3-Piperidinedicarboxylic acid (*cis*-2,3-PDA, **1**), *trans*-2,3-PDA (**2**), and *trans*-2,4-PDA (**4**) were purchased Tocris Cookson Ltd. and were used as received. *trans*-2,4-PDA (**3**), *cis*-2,5-PDA (**5**), and *trans*-2,5-PDA (**6**) were synthesized[13] by Dr. Ulf Madsen, Royal Danish School of Pharmacy, Copenhagen.

NMR Titrations. A PDA (20 mg) was dissolved in 0.6 mL of H_2O containing 10% D_2O and alkalized with NaOH (0.5 or 0.1 M). All solutions were buffered with KCl to a ionic strength of 1 M. The sample was then titrated with HCl (0.5 or 0.1 M), recording $^{13}C\{^1H\}$ NMR spectra for each pH change of 0.2 units. pH of the sample was measured directly in the NMR tube before and after each spectrum, and a mean value was adopted for the calculations. The pH measurements were performed at ambient temperature with a MI-412 microcombination electrode from Microelectrodes, Inc. (Bedford, NH) attached to a PMH220 laboratory pH-meter from Radiometer (Copenhagen, Denmark). Two-point calibration was achieved using certified buffer solutions (Radiometer), and the pH readings are believed to be accurate to within 0.05 pH unit. The spectra were recorded with a Bruker AMX400WB spectrometer operating at 100.62 MHz for ^{13}C. Chemical shifts were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). All spectra were recorded at 298 K. Macroscopic pK_a values were derived from the data by simultaneous nonlinear fitting of the experimental ^{13}C NMR titration curves for all carbons to a set of equations represented by eq 1 using GraFit v. 4.0.[14,15] In eq 1, K_1, K_2, and K_3 are equilibrium constants for successive deprotonation of fully protonated species, δ_i^n (n = 0, 1, 2, and 3) are the intrinsic chemical shifts of nonprotonated, monoprotonated, diprotonated, and triprotonated (fully protonated) species, and δ_i is the observed chemical shift of the ith carbon of the molecule.

$$\delta_i = \frac{\delta_i^0 K_1K_2K_3 + \delta_i^1 K_1K_2[H^+] + \delta_i^2 K_1[H^+]^2 + \delta_i^3[H^+]^3}{K_1K_2K_3 + K_1K_2[H^+] + K_1[H^+]^2 + [H^+]^3} \qquad (1)$$

Assignment of chemical shifts of the two carboxylic groups in **1**−**6** has been done by consideration of the previously reported pK_a values for glutamate, pK_{a1} = 2.23, pK_{a2} = 4.37, and pK_{a3} = 9.53,[16] with pK_{a1} of glutamate corresponding to deprotonation of the α-carboxylic acid.

Computational Details. Structures were optimized at the HF/6-31+G* level, employing the conductor version of PCM model[17,18] with UAHF radii[19] as implemented in Gaussian98.[20] All structures were fully

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Table 1. Experimentally Determined pK_a Values with Standard Errors

compd	pK_{a1}	pK_{a2}	pK_{a3}
1	1.73 ± 0.02	3.60 ± 0.01	12.11 ± 0.00
2	1.65 ± 0.02	3.92 ± 0.01	10.54 ± 0.00
3	1.96 ± 0.01	3.85 ± 0.00	10.27 ± 0.00
4	1.99 ± 0.01	4.01 ± 0.00	10.45 ± 0.00
5	1.78 ± 0.02	3.48 ± 0.01	10.39 ± 0.01
6	1.75 ± 0.02	3.56 ± 0.01	10.05 ± 0.00

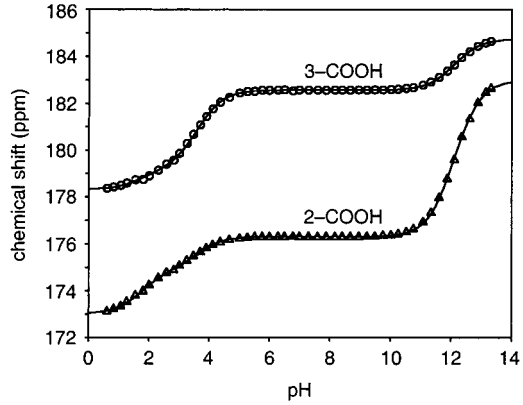

Figure 3. ^{13}C NMR titration curves for carboxylic group carbons in compound **1** (10% D_2O in H_2O, 298 K). The points represent experimental data, and the curves are the best fits through the data.

relaxed within the solvent model; gas-phase geometries were not employed at any point. Total free energies are reported as calculated, that is, including solvation at the default temperature of 298.15 K, but not other terms such as, e.g., vibrational contributions. For each compound, the two chair conformers were investigated, i.e., with the 2-carboxylate group in equatorial or axial position. The neutral states of the functional groups required investigation of the conformational forms with respect to axial vs equatorial position of the amine hydrogen, as well as rotamers of the carboxylic acid groups. All possible conformations were subjected to full optimization. The possibilities of twist-boat conformations and alternative conformations of the carboxylic acids were investigated in a few test cases (identified by maximum probability of forming stabilizing interactions) but were in all cases found to possess much higher total energies. The reported ensemble free energy for each compound was calculated by summation over all contributing conformers according to eq 2. In addition, a constant of *RT* ln 2 was added for each ionic carboxylate moiety to the ensemble free energy to account for degenerate rotameric forms. The ΔG_i are total free energies in solution for each unique conformation as reported by Gaussian.

$$\Delta G_{tot} = -RT \ln[\Sigma_i \exp(-\Delta G_i/RT)] \quad (2)$$

The theoretical relationship between pK_a and the free energy difference between protonated and nonprotonated species of any type is given by eq 3. The intercept which corresponds to the free energy of the solvated proton can be calculated, but to avoid a strong influence from systematic basis set deficiency errors, it was chosen to regard it as a regression parameter. However, deviations from the slope defined by eq 3 can be used to identify systematic errors in the calculations.

$$\Delta\Delta G = \Delta G(AH^+) - \Delta G(A) = RT \ln 10 \cdot pK_a + \Delta G(H^+) \quad (3)$$

Results

The three macroscopic pK_a values determined for each of the compounds **1**−**6** are shown in Table 1, and an example of the titration curve in Figure 3. All titration results are included as Supporting Information.

Table 2 gives the relative ensemble energies calculated according to eq 2 for each protonation state of **1**−**6**. Note that,

Table 2. Calculated Ensemble Energies (kcal/mol) for the Deprotonations **f** → **d**, **d** → **c**, and **c** → **b**

compd	Δ*G*(**d**−**f**)	Δ*G*(**c**−**d**)	Δ*G*(**b**−**c**)
1	279.6	283.7	302.3
2	279.2	284.4	298.9
3	279.3	285.6	297.0
4	280.0	285.4	297.4
5	279.9	283.6	299.7
6	280.0	284.0	298.2

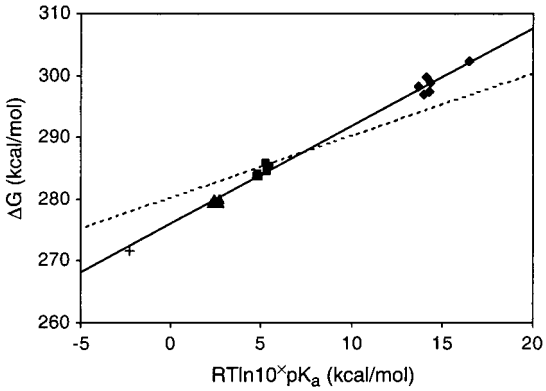

Figure 4. Correlation between calculated free energies of deprotonation and experimental pK_a values converted into free energies. Calculated energies for the deprotonations **f** → **d**, **d** → **c**, and **c** → **b** are displayed by ▲, ■, and ◆, respectively; + denotes the calculated free energy of deprotonation of H_3O^+.

for the zwitterionic states, deprotonation of each carboxylic acid group has been considered separately (**d** and **e**, Figure 2). The experimental as well as the calculated results clearly indicate that the 2-carboxylic acid in all cases is the most acidic one, as expected from the proximity to the ammonium moiety. The maximum stability of the **e** isomer can be found for structure **1**, but the calculated equilibrium population of **1e** is still only about 1%. The contributions from isomer **e** have therefore been ignored. The possibility of initial protonation of a carboxylate in lieu of the amine (protonation state **a**, as would be expected in the gas phase) has also been ignored. This corresponds to the treatment of the experimental, macroscopic pK_{a1-}pK_{a3} values as essentially equivalent with microscopic pK_a values of the α-carboxylic group, the second carboxylic group, and the ammonium group, respectively.

A correlation between the experimental pK_a values shown in Table 1 and calculated ensemble energy differences in Table 2 is shown in Figure 4. The linear regression is shown as a solid line, while the theoretically expected slope is indicated by a dashed line.

Discussion

The PCM−UAHF model employed in the current work has previously been shown to give a correct ordering of the pK_a values for a series of monocarboxylic acids.[7] However, since the estimated regression coefficient was found to be about twice the theoretically expected value, the results indicate some remaining systematic error. As shown in Table 1 and Figure 4, the pK_a values for the carboxylic acid groups in **1**−**6** fall into two distinct groups depending on the strength of the interaction with the ammonium moiety. Regression through the data points corresponding to the deprotonation of the two carboxylic acid groups gives a correlation coefficient $r^2 = 0.97$ with a slope of 1.82 ± 0.11 (theoretically expected as 1.0). Thus, despite the fact that the deprotonations of the carboxylic acids investigated

in this study occur in zwitterionic or cationic compounds containing possible internal hydrogen bonds, our results are similar to those of Schüürmann et al.[7] This observation suggests that the PCM−UAHF model is able to account for strong intramolecular and solvent−solute electrostatic interactions.

In general, most computational pK_a studies are related to the deprotonation of carboxylic acids and thus restricted to a narrow pK_a range (i.e., 2.4 pK_a units for the PDAs). By inclusion of the deprotonation of the ammonium moiety of the PDAs, the pK_a coverage in the present study is expanded to a range of 10.5 pK_a units. The correlation between the calculated relative energies and the experimental pK_a values including all data points gives $r^2 = 0.99$ with a slope of 1.58 ± 0.03 (Figure 4). Thus, the slope is larger than the theoretically expected value of 1.0, but this is consistent with other related studies.[7]

To verify the results and to extend the range of the study, the pK_a value of H_3O^+ (the definition of the pK_a scale) was calculated by the same methods and found to fall almost exactly on the regression line (Figure 4).

In the different protonation states and calculated low-energy conformations of **1**−**6**, the ammonium or amine group may form one or two hydrogen bonds to the carboxylate/carboxylic acid moieties. These hydrogen bonds differ considerably in strength ranging from a very strong ion pair to a much weaker neutral amine−carboxylic acid hydrogen bond. It is therefore gratifying to note that relative stabilities of different protonation states of the PDAs can be predicted accurately. The maximum deviation from the regression line is only 1.3 kcal/mol, with an rms deviation of 0.7 kcal/mol. Moreover we find it promising that a prediction of relative pK_a values of compounds possessing two different types of ionizable functional groups covering a pK_a range from 1.6 to 12.1 is possible.

The most noticeable result in Table 1 is the pK_{a3} value for compound **1** of 12.11, which is 1.6−2.0 pK_a units higher than the corresponding values for **2**-**6**. This is nicely accounted for by the calculated energies (Table 2) and the almost perfect correlation shown in Figure 4. The free energy difference ΔG(**b**−**c**) is calculated to be significantly larger for **1** than for the compounds **2**−**6**. For the latter compounds, the pK_a differences are found to be within 0.5 pK_a units and the calculated ΔG(**b**−**c**) energy differences are similar.

Analysis of the calculated total energies for **1**−**6** (see Supporting Information for listings of energies) shows that the total energies (including hydration energies) for the most stable conformers of the triply ionized **1c**−**6c** are very similar for all compounds (±1 kcal/mol with respect to **1c**). Irrespective of differences in type and number of internal hydrogen bond interactions and conformational properties (axial/equatorial, gauche/anti carboxylate groups), the differences in internal energies (total energy minus hydration energy) are virtually completely counterbalanced by differences in free energies of hydration. For example, the calculated internal energy of the lowest energy conformation of **1c** is 9.8 kcal/mol higher than that of the trans isomer **2c** due to gauche dianion repulsion in **1c**. This energy difference is counterbalanced by a more negative free energy of hydration of **1c** by 10.9 kcal/mol. The net energy difference is thus only 1.1 kcal/mol in favor of **1c**. Similarly, the counterbalancing of the internal energy difference and the difference in solvation energies results in a net total energy difference between **1c** and **6c** of only 0.6 kcal/mol.

In contrast, the N-deprotonated form **1b** is significantly less stable than **2b**−**6b**. The calculated internal energy for the lowest energy conformation of **1b** is higher than those of **3b**−**6b** by 17−25 kcal/mol. These energy differences are reduced by the significantly more negative free energy of hydration of **1b** by 14−20 kcal/mol. The energy differences are smaller when comparing **1b** and **2b**. The internal energy of **1b** is higher by only 1.3 kcal/mol, whereas the free energy of hydration of **1b** is more positive by 1.2 kcal/mol. In comparison with **2b**−**6b**, the net effect is a higher energy of **1b** in aqueous phase by 2.5−5.9 kcal/mol.

The similar total energies of **1c**−**6c** and the higher total energy of **1b** as compared to **2b**−**6b** results in a significantly larger ΔG(**b**−**c**) for **1** than for **2**−**6**. Thus, the higher pK_{a3} value for **1** than for **2**−**6** is largely due to the lower stability of the N-deprotonated form **1b** compared to **2b**−**6b**. Compound **1b** is the only compound among those studied in this work which contains both one axial substituent and the two substituents in gauche conformation in all possible chair conformations.

The pK_a values of the 3-, 4-, and 5-carboxylic acid groups (pK_{a2} in Table 1) are all within 0.5 pK_a units. These observations are in agreement with the calculated ΔG(**c**−**d**) values which are very similar for all compounds studied (Table 2). The largest energy difference is 2.0 kcal/mol, which should be compared with the largest ΔG(**b**−**c**) difference of 5.3 kcal/mol. The absolute total energies of protonation state **c** and **d** change very little from one compound to the other. All energies are within 1.3 kcal/mol with the exception of **3d**, which is calculated to be lower in energy than **1d** by 2.5 kcal/mol. Compounds **2**−**4** have the highest pK_{a2} values and accordingly the largest calculated ΔG(**b**−**c**) values.

The pK_a values for the 2-carboxylic acid group in **1**−**6** (pK_{a1} in Table 1) are also very similar. The largest difference is 0.3 pK_a units. In agreement with this, the calculated ΔG(**d**−**f**) are also virtually identical, with a largest total energy difference of 0.8 kcal/mol (Table 2).

Conclusions

Calculation of properties of the highly ionizable piperidine dicarboxylic acids (PDAs) in water represents a major challenge to current computational methods. The compounds are of interest as conformationally restricted α-amino acids. It has been shown here that the PCM model applied at the HF/6-31+G* level is able to calculate relative stabilities of all protonation states with good accuracy and to give successful predictions of relative pK_a values for two different types of ionizable functional groups (carboxylic acid and amine), which cover a pK_a range from 1.6 to 12.1. Due to the restrictions of the number of attainable conformations and the variety of substitution patterns available, the PDAs form an interesting test set for theoretical solvation models.

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Supporting Information Available: Figures showing ^{13}C NMR titration data for compounds **1**−**6 and** tables of calculated total energies and free energies of solvation for **1b**−**6b**, **1c**−**6c**, **1d**−**6d,** and **1f**−**6f**. This material is available free of charge via the Internet at http://pubs.acs.org.

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