Unusual Properties of Highly Charged Buffers: Large Ionization Volumes and Low Barrier Hydrogen Bonds

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Abstract: Pressure effects on the ionization of various compounds were determined by pressure-modulated fluoresence spectroscopy of buffering solutions containing nanomolar fluorescein as a pH indicator. Two highly pressure-sensitive buffers are described. The second ionization of 1,1-cyclopropane dicarboxylic acid (pK 7.5) has a ΔV_{ion} of -29 ± 2 mL/mol, the largest known aqueous ionization volume of any Brönsted acid of pK_a below 9. The third ionization of 1,4,8,12-tetraazacyclopentadecane exhibits a ΔV_{ion} of $+30 \pm 2$ mL/mol, the largest positive aqueous ionization volume known for any Brönsted base in water. The ^{1}H NMR spectrum of the tetrabutylammonium salt of 1,1-cyclopropane dicarboxylic acid monoanion in acetone-d_6 with 10% H_2O at 25 °C had a peak at 19.25 ppm, suggesting that the two carbonyl groups are tethered by a low-barrier hydrogen bond which shields electron density from the solvent and that ionization of this proton leads to a large increase in solvation of the carboxylates. The protonation of 1,4,8,12-tetraazacyclopentadecane dication causes a structural change that is accompanied by a large increase in charge density and may contribute to the large ionization volume via enhancement of electrostriction.

Introduction

The effects of hydrostatic pressure on the pH of buffered aqueous systems have often been investigated with an eye toward finding pressure-insensitive buffers for use in experimental investigations on the effects of pressure on aqueous chemical and biochemical systems. We are interested in buffer systems with great pressure sensitivity and their potential uses, so we chose compounds for study that promised to possess large ionization volumes. The pressure-sensitive compounds that we identified have interesting chemical properties that may have a relationship to their sensitivity to pressure.

Pressure effects on buffers are related to the volume of ionization of that compound, which is defined as the difference in partial molar volumes of the products and reactants of the buffer's ionization reaction. Carboxylic acids have negative ionization volumes whereas bases such as amines generally have positive ionization volumes. The volume change comes primarily from solvent electrostriction, i.e., the tendency of a polar solvent to adopt a higher density configuration around an ion.[1] Elevating the hydrostatic pressure lowers the free energy of ion solvation and perturbs the equilibrium toward the ionized form. Buffers with high charge density have larger absolute ionization volumes, e.g., the volumes for the second, third, and fourth ionizations of pyrophosphate are −16, −21, and −29 mL/mol, respectively.[2] The presence of internal hydrogen bonding is also associated with large absolute ionization volumes: the volume of the second ionization of maleate is −24 mL/mol, whereas the second ionization of t-aconitate has an ionization volume of −16 mL/mol.[2,3] The anion of maleic acid is known to possess a downfield shift of 20.2 ppm in 90% acetone/10% H_2O at −50 °C,[1] characteristic of a low barrier hydrogen bond (LBHB). Absolute ionization volumes are decreased when charge is delocalized, as in an aromatic buffer. Sterically hindered bases possess elevated ionization volumes,[4] possibly due to rearrangement of solvent around the ion in a form that is highly energetically unfavorable in the neutral form.

The decreased ionization volume observed in strong hydrogen bonding systems is a potential tool in the study of low barrier hydrogen bonds (LBHBs). Strong hydrogen bonds have recently been proposed[5−7] to be important in enzyme catalysis. Observation of LBHBs may indicate the presence of a short strong hydrogen bond. LBHBs are characterized by low fractionation factors, particular deuterium isotope effects on NMR chemical shifts and IR stretching frequencies, and downfield proton chemical shifts. A LBHB proton is believed to dwell centrally between the donor and acceptor heteroatoms with little barrier to transfer between potential wells. In weaker, longer hydrogen bonds the proton resides primarily on one atom.

LBHBs have been proposed[6] to increase the rate of enzymatic catalysis in some enzymes by stabilization of intermediates by as much as 10−20 kcal/mol. Chemical models of enzymes have yielded evidence for LBHB participation in hydrolytic reactions catalyzed by chymotrypsin. Calorimetry measurements of the heat of formation of alkylimidazole−carboxylic complexes, chemical models of the enzyme His-Asp interaction, ranged from 12 to 15 kcal/mol.[8] Deuterium isotope effects have been observed on the antisymmetric carbonyl stretch of these complexes.[9,10] Downfield proton NMR shifts (19−18.3 ppm)

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are observed in both model complexes[9,10] and in transition state analog–enzyme complexes.[11–14] Low fractionation factors (0.4)[15] and elevated basicity[11,12] of the $N^{\epsilon 2}$ in His57 are found for transition state analog–chymotrypsin complexes.

Some disagreement exists as to whether the energy derived from a LBHB can be as large as 10–20 kcal/mol in the aqueous environment of an enzyme.[16] For instance, hydrogen bonds to water may interrupt binding in the transition state of an enzymatic reaction. Electrostatics alone have been proposed[17,18] to account for the hydrogen-bonding energy. Since ionization and activation volumes of catalyst are sensitive to hydration states, molar volumes or pressure effects may be a useful tool for addressing the presence and enzymatic contributions of LBHBs.

Studies of ionization volumes to discover pressure-sensitive buffers have both scientific and technological utility. It is desirable to have control of free-ion concentrations, so that the equilibrium position of a reaction may be modified cyclically or rates of one or more competing reactions may be altered in a cyclical manner. One example is an isothermal polymerase chain reaction (PCR) in which a pressure-induced pH change causes cyclical denaturation of double-stranded nucleic acid. Another potential application is control of the copolymerization of two or more different monomers, where the rate of addition of each type of monomer is dependent on free-ion concentration such as pH and each type of monomer has a different pH optimum for rate of addition; the pattern of pressure cycling can then be used to control the properties of the resulting polymer. In some cases, control of the reaction can be accomplished through activation or deactivation of a pH-sensitive catalyst. Alteration of pH, pOH, or other buffered ions through cyclical alteration of pressure is a much more practical alternative than continuous additions of salts to modify free-ion concentrations since there is no accumulation of salts and the process may be rapidly modulated. An ion exchange support with pressure-sensitive binding groups could lead to improved separations through pressure modulation.

Our goal in the present study was to find buffers with large ionization volumes whose characteristics could then be used to design more pressure-sensitive buffering systems. Compounds **1** and **2** have a high charge density and were selected for investigation.

Experimental Section

All measurements were done at 25 °C, with the exception of some NMR experiments, as noted. Fluorescein, 1,4,8,12-tetraazacyclopentadecane, 1,1-dicarboxylic cyclopropane, cacodylic acid, 3,3-dimethylglutaric acid, imidazole, 2-[*N*-morpholino]ethanesulfonic acid (MES), bis-tris propane, sodium pyrophosphate, potassium bicarbonate, and spectroscopic grade ethanol were from Aldrich. Highly fluorescent contaminants of 1,4,8,12-tetraazacyclopentadecane were removed by addition of 0.1 g of charcoal to 50 mL of the pH adjusted buffer solution, followed by centrifugation.

Table 1. Buffers of Known Ionization Volumes[2]

compd	ionization vol
bis-tris propane	10.5
imidazole	1.8
cacodylic acid	−13.2
pyrophosphate	−20.7
3′,3′-dimethylglutarate	−25.0
bicarbonate	−27.6

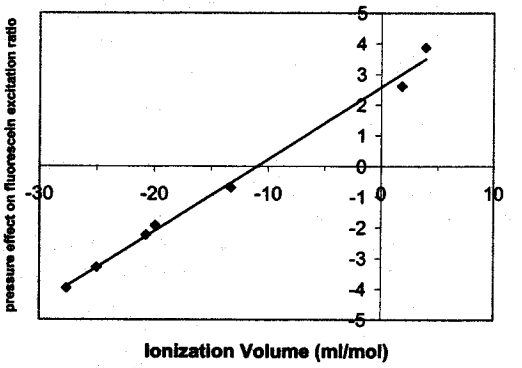

Figure 1. A linear regression of the plot of the pressure sensitivity in the fluorescein excitation ratio vs the ionization volumes of the standards gave a slope of 1.606×10^{-6} ratio units·psi^{-1}·(mL·mol^{-1}) and a y intercept of 1.35×10^{-5} ratio units·psi^{-1}. Pressure sensitivities were determined by measurement of the ratio of fluorescence intensity with stimulation of fluorescein by 490 and 450 nm light. This ratio was determined at several pressures from 1 to 1.5 MPa The x intercept was −8.4, a measurement of the ionization volume for fluorescein phenol.

Measurement of Ionization Volumes. Ionization volumes were measured using fluorescein as a pH probe inside of a high-pressure bomb with sapphire windows mounted in a fluorometer. The ratio of fluorescence intensity (emission at 520 nm) for excitation at 490 and 450 nm measures the pH due to the loss of fluorescence intensity from the 490 nm excitation upon protonation of the phenol. The 450 nm excitation probes the fluorescein carboxylate, which will remain unprotonated and acts as an internal intensity standard. The pH shifts in response to pressure do to changes in the pK_a of the buffer being tested. This pK_a shift is proportional to the ionization volume of the buffer. The concentration of fluorescein (nM) needed is negligible compared to the buffer concentration (mM) and will not perturb the pH. Buffer standards (Table 1) were made to 50 mM, with a pH of between 5.1 and 6.5 and with the pH being no more than 0.6 units from the pK of the buffer. Fluorescein was added to a concentration of 45 nM by addition of 75 μL of 900 nM stock solution to 1425 μL of buffer. The sample was mixed well and loaded into a quartz bottle (ISS; Champaign, IL). A polyethylene cap was firmly placed on the bottle so that it overlapped the bottle's neck by several millimeters. The bottle was placed in an ethanol-filled, high-pressure spectroscopic cell with sapphire optical windows (ISS). The cap of the pressure cell was secured with a 50 ft·lb torque wrench, placed into an ISS PC1 fluorometer and attached by a flexible metal hose to the hand-cranked pressure generator (High Pressure Equipment, Erie, PA) equipped with a pressure transducer (Sensotec). Spectroscopic grade ethanol was used as the pressurizing medium. The ratio of excitation at 490 to 450 nm was measured with a 4 nm band-pass on the excitation monochromator and observed by monitoring emission at 520 nm with a 16 nm band-pass. Measurements were made every 34 MPa to 134 MPa. The excitation ratio vs pressure (pressure-sensitivity) was plotted versus pressure and the slope was estimated by regression. Precaution was taken to only use measurements for which the excitation ratio may be approximated as a linear function of pH, which was taken to be from 1.8 to 3.6. The pressure sensitivity for each standard buffer was determined at least three times. The average values were used to make a standard curve (Figure 1) by plotting the pressure sensitivities vs ionization volumes of buffers for which the ionization values are known (Table 1).

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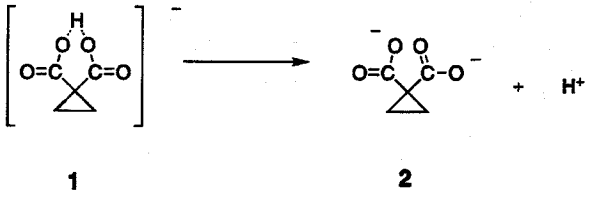
Table 2

buffer	pK	pH	ΔV_{ion} (mL/mol)
1,1-dicarboxylic acid	7.2	6.5	-29 ± 2 ($m = 3$)
1,4,8,12-tetrazacyclopentadecane	4.8	5.1	$+30 \pm 2$ ($m = 3$)

Scheme 1



1 **2**

The buffers of unknown ionization volumes were prepared as sodium salts to 50 mM. For each unknown buffer, the pressure sensitivity was determined three times and the ionization values were determined from the equation derived from a linear regression of the standard curve. The errors in the unknowns were determined using the equation[23]

$$s_{x_0} = \frac{s_{y/x}}{b} + \left[\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})}\right]^{1/2}$$

where y_0 is the experimental value of y from which the ionization volume x_0 is to be determined, s_{x_0} is the estimated standard deviation of x_0, $s_{y/x}$ is the standard deviation of the slope of the standard curve (b), n is the number of points on the standard curve, and m is the number of readings for the unknown.

pK_a Determinations. The pK_a values, as determined by pH titration, of 1,4,8,12-tetraazacyclopentadecane are 3.1, 4.8, 9.7, and 11.0. The pK_a values of 1,1-cyclopropane dicarboxylic acid are 2.1 and 7.2.

NMR. 1,1-Cyclopropanedicarboxylic acid (0.5 g, 3.25×10^{-3} mol) was added in one portion to 1 M tetrabutylammonium hydroxide (3.25 mL) in methanol under nitrogen. The mixture was stirred for 15 min and the solvent was removed under vacuum. The white solid (1.1 g, 92%) was dried under vacuum at 110 °C in the presence of P_2O_5. ^{1}H NMR chemical shifts of the tetrabutylammonium salt of **1** were determined at 200 or 500 MHz in 90% acetone/10% H_2O. The purity of the compounds was confirmed by ^{13}C NMR.

Results

The 490:450 fluorescence excitation ratio of fluorescein was observed to be pressure sensitive in the presence of compunds with large ionization volumes. The pressure dependence of the fluorescein excitation ratio was highly linear to 134 MPa. By constructing a standard curve of the pressure effects on this excitation ratio, one can determine the ionization volume of unknown compounds. The ionization volumes of **1** and **2** were determined from their pressure sensitivities using the standard curve and are listed in Table 2. The standard curve is well described by a linear relationship between the pressure sensitivity of the fluorescein excitation ratio and the reported ionization volumes (Table 1) of the compounds tested. Buffering effects of the fluorescein are easily neglected, as its concentration is 10^6-fold lower than the concentration of buffer. The results of one standard, bis-tris-propane, were discarded due to anomalously high values of the fluorescein excitation ratio which are consistent with full deprotonation of fluorescein and likely to arise from stabilization of the fluorescein dianion by the dicationic buffer. The charged groups of these two compounds are favorably oriented for interaction.

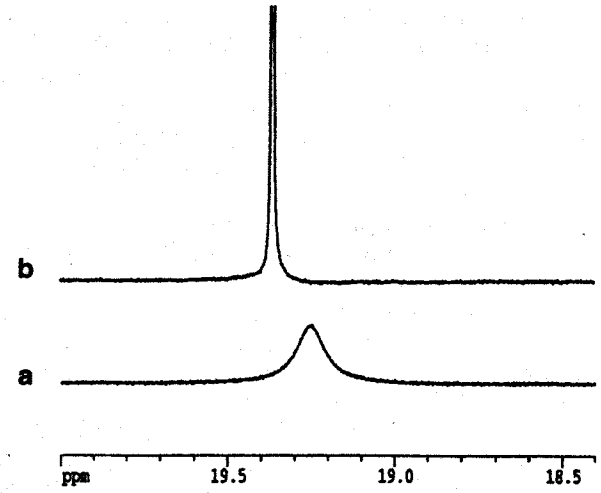


Figure 2. ^{1}H NMR spectra of the tetrabutylammonium salt of 1,1-cyclopropanecarboxylic acid in acetone-d_6 (90%)/H_2O (10%): (a) 25 and (b) −40 °C.

NMR Results. In the ^{1}H NMR spectra, the carboxylic acid proton of the monoanion of the tetrabutylammonium salt of cyclopropanedicarboxylic acid has a chemical shift of 19.3 ppm in acetone-d_6. This shift is far downfield (~7 ppm) from that of carboxylic acid protons which are not strongly hydrogen bonded. In a solvent mixture of acetone-d_6/H_2O (90:10) at 25 °C the carboxylic acid peak is broadened due to exchange with water and is shifted to 19.25 ppm as shown in Figure 2. Lowering the temperature to −40 °C slows the exchange and sharpens the peak (19.36 ppm) considerably (Figure 2).

Discussion

The large ionization volumes observed for **1** and **3** are likely to be due to a combination of their high charge densities, intramolecular hydrogen bonding, and conformational changes and lack of electron delocalization.

The pK_a of 2.1 for 1,1-cyclopropane dicarboxylic acid is much lower than the pK_a values of typical carboxylates (e.g., 4.76 for acetic acid), and suggests a strong intramolecular hydrogen bond exists in aqueous solutions of compound **1**. Dianion **2** has a high charge density, but the trianion of pyrophosphate has a higher charge density and similar ionization volume. This suggests the possibility that the intramolecular hydrogen bond that is broken upon ionization makes a contribution to the ionization volume beyond what would be expected due to charge density effects. The 2.41 Å hydrogen bond seen in the X-ray crystal structure[24] and the downfield ^{1}H NMR shift of 19.25 ppm in 10% acetone−water at 25 °C suggest an extremely strong hydrogen bond. The observation of the NMR peak at room temperature indicates a very slow exchange rate with the solvent, and may be another indicator of hydrogen bond strength. The hydrogen bonding properties of the compound are a result of steric constraints due to the cyclopropane ring and underscore a problem with suggestions that hydration of enzyme active sites precludes the role of LBHBs in enzyme catalysis; steric influences of the enzyme structure can force a close interaction

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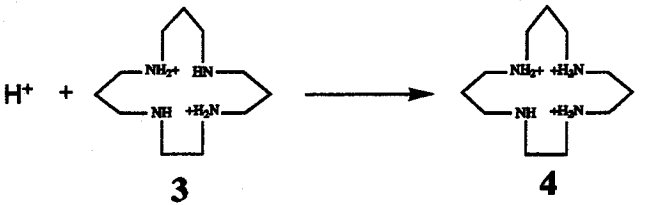
with the substrate. However, observation of a downfield shift in an enzyme's ^{1}H NMR spectra may in some cases only prove that a hydrogen bond donor and acceptor are closely positioned due to the protein structure. Suggestion of a role in catalysis should be limited to those situations where the putative LBHB only exists in the transition state or to an intermediate.

The NMR downfield shift and large ionization volume for **2** raise the possibility that the two phenomenon are related. The electronic shielding of the proton is due to electron delocalization across the short hydrogen bond between the carboxyl moieties. Upon ionization, this electronic delocalization is lost and the full negative charge is available for interaction with solvent, causing an increased electrostriction effect. Simultaneously, the two carboxylates will re-orient such that there is greater solvent accessibility to the two groups. It is not known to what degree the hydrogen bonding and conformational and charge density changes contribute to the unusually large ionization volume of this compound. If the protonation of the dianion of 1,1-cyclopropane dicarboxylic acid is considred as a model for formation of an LBHB at an enzyme active site, one may imagine that the enzyme had evolved to have a global structure that energetically compensated for an unstable electrostatic interaction in the active site that is relieved upon formation of an LBHB. Thus LBHBs may in some cases be a result of ground-state destabilization and further investigation may shed light on such mechanisms of enzyme action. Compound **3** will adopt a conformation that mimimizes charge repulsions. Upon protonation, **3** will be restricted in its ability to minimize charge repulsion, causing **4** to have a much higher charge density than **3**. The conformation of **4** will occupy a smaller volume and may allow a higher degree of solvation than

Scheme 2



3. In contrast, double protonation of diethylenetriamine causes a volume change of only 28.8 or 9.6 mL/mol per ionization,[22] despite a comparable charge density. The combination of structural and solvation changes upon a single protonation of **3** appears to give rise to an ionization volume of 30 mL/mol, larger than any (to our knowledge) previously reported value for a single ionization in aqueous media. A solution buffered by this compound may decrease in pOH by greater than 2.5 units upon application of 400 MPa of pressure. The possibility exists that high charge density causes the pressure effect on the fourth protonation to be sufficiently large as to raise the pK of this group from 3.1 to greater than 6, although no nonlinearity in the effect of pressure on pH was observed.

Knowledge of the contribution of hydrogen bonding to ionization volumes may be useful as a tool for answering questions related to the role of low-barrier hydrogen bonds in solution and enzymatic chemistry. The high volumes of strongly hydrogen bonded transition states relative to the ionized forms may result in positive or less negative activation volumes for enzyme reactions that involve LBHBs.

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