

Salt Bridges investigated by NMR

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Salt bridges are interactions, electrostatic combined with hydrogen bonding, between oppositely charged residues, typically carboxylic acid anions and ammonium ions, provided they are close together. For an illustration see Fig. 1. Salt bridges are of particular interest in proteins and other biomolecules. In the present contribution salt bridges are investigated by means of ^1H chemical shifts, determination of pKa values and deuterium isotope effect on ^{15}N and ^1H chemical shifts. In the latter case model compounds like ammonium ions are also investigated and the use of deuterium isotope effects on chemical shifts are supported by Density Functional Theory (DFT) calculations. The use of isotope effects on chemical shifts enables a distinction between salt bridges observed in the solid state by X-ray diffraction and those actually present in solution.

Keywords: deuterium isotope effects on chemical shifts ; salt bridges ; proteins ; theoretical calculations

1. Introduction

Salt bridges may contribute to protein stability. They may occur both in the interior and at the surface.

Salt bridges have traditionally been investigated by measuring changes in pKa values, the mutation approach, X-ray studies of crystalline samples. These techniques are reviewed. ^[1]

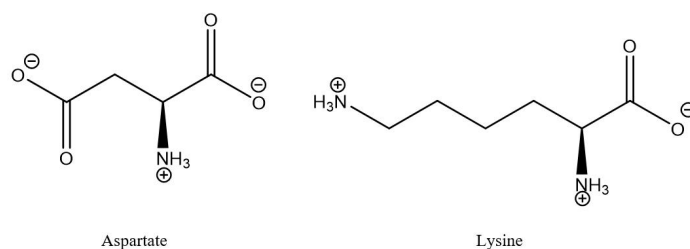


Figure 1. Typical salt bridge between aspartic acid and lysine. The aminoacids may typically be part of different strands of proteins

Salt bridges in the present paper are investigated using ^1H NMR chemical shifts, determination of pKa values by NMR and deuterium isotope effects on ^{15}N and ^1H chemical shifts. In the latter case model compounds like ammonium salts are investigated and the use of isotope effects on chemical shifts are supported by Density Functional Theory (DFT) calculations (see later). The use of isotope effects on chemical shifts enables a distinction between salt bridges observed in the solid state by X-ray diffraction and those actually present in solution.

2. NMR Titration Studies

A classic case is that of Bovine Pancreatic Trypsin Inhibitor (BPTI) in which the terminals (the C- and the N-terminal) forms a salt bridge in solution, but not in the crystal state. This was demonstrated early on by Wüthrich and co-workers. ^[2]

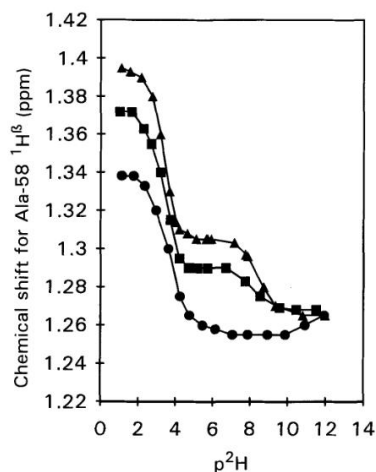


Figure 2. ^1H chemical shifts vs $p^2\text{H}$ for the C-terminal Alanine 58 H- α resonance in WT-BPTI (triangles), 1-BPTI (squares) and 4-BPTI (dots). Samples contained 2 mM protein in 0.3 M KCl and spectra were recorded at 25°C, 250 MHz. From Ref. [3] with permission from Elsevier.

The idea of determining the pKa values is, that for the acid, a drop in pKa value is expected, if a salt bridge is formed. This is indeed seen in Fig. 2. For the WT-BPTI the value is 3.33, and for 1-BPTI the pKa value is 3.35, whereas for 4-BPTI, not being able to form a salt bridge, it is 3.67. 4-BPTI has four extra amino acids at the N-terminus. [3] This picture is confirmed for 3-58BPTI missing to residues at the N-terminus and not being able to form a salt bridge. In that case the pKa value is 3.72. [4]

The pKa values of e.g. can also be determined by NMR. In that case an increase of the pKa value is expected. For the lysines of protein G B1 domain this was not found in line with the finding (see later), that no salt bridges with lysines exist in this protein in solution. [5]

3. NH Chemical Shifts

The formation of a salt bridge in the complex between dihydrofolate reductase and methotrexate, brodimoprim 4-carboxylate or brodimoprim-4,6-dicarboxylate was established between the ligand and Arginine 57 based on the finding of a higher NH chemical shift for those NH protons being part of the salt bridge compared to those not. [6]

4. Use of Isotope Effects on Chemical Shifts to Study Salt Bridges

4.1. Isotope effects on chemical shifts

Exchange of a ^{15}NH with a deuterium leads to a change in the ^{15}N chemical shift as illustrated in Fig. 3. Upon deuteration the vibration sits lower in the potential well. This leads on the average to a shorter ND bond, which as seen in Fig. 3b leads to a change in the ^{15}N nuclear shielding (equal to a smaller chemical shift). The difference between the chemical shift of the deuterated species minus the protio one is called the isotope effect, $^n\Delta(\text{D}) = \delta\text{X}(\text{H}) - \delta\text{X}(\text{D})$. If n is one it is called a one-bond deuterium isotope effect. If X is nitrogen, $^1\Delta\text{N}(\text{D})$. A negative two bond deuterium isotope effect is observed at the hydrogens, $^2\Delta\text{H}(\text{D})$. As isotope effects are measured as a difference in chemical shifts in the same solution, they can be measured very accurately. A prerequisite is of course that separate signals can be observed. For reviews on isotope effects on chemical shifts. [7]

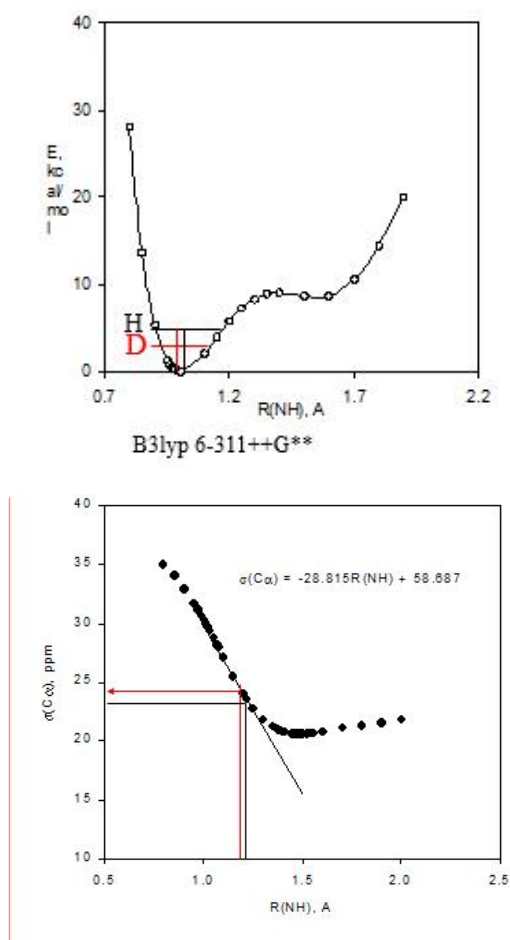


Figure 3. a. Potential energy well. B3lyp 6-311++G** shows the DFT functional and basis set used. b. Calculated ^{15}N nuclear shielding surface vs. NH bond length.

4.2. Use of model compounds

Ammonium ions are a close analogue of e.g. the side chain of protonated lysine. It was shown that one-bond deuterium isotope effects on ^{15}N chemical shifts, $^1\Delta\text{N}(\text{D})$, depended strongly on the counter ion, the counter ion concentration and the smallest $^1\Delta\text{N}(\text{D})$ were found in very dilute solutions Fig. 4. A similar picture was seen for $^2\Delta\text{H}(\text{D})$. [8]

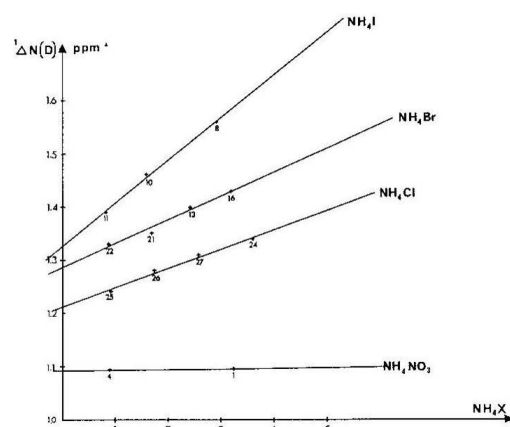


Figure 4. Plot of one-bond deuterium isotope effects on ^{15}N chemical shifts vs. concentration of ammonium salt. Taken from Ref. 7 with permission from the Danish Chemical Society

Furthermore, if the counter-ion was far away, ammonium ion buried in a cryptand like SC-24, no effect of the counter ion was found. [9] In addition, a solid state study showed that $^1\Delta\text{N}(\text{D})$ decreases as the heavy atom distance decreases [10] and this was confirmed by theoretical calculations. [11] To sum up, $^1\Delta\text{N}(\text{D})$ will be smaller, if a salt bridge is present, whereas $^2\Delta\text{H}(\text{D})$ will become more negative.

5. Salt Bridges in Proteins

Salt bridges can both be "buried", in the inner part of the protein, or solvent exposed at the surface. Exchange of the NH protons can occur. A useful pulse sequence to overcome exchange problems is , HISQC ^[12] A typical spectrum is seen in Fig. 5.

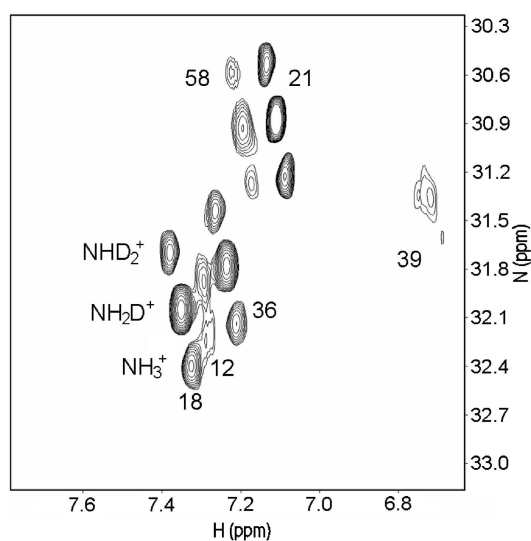


Figure 5. HISQC spectrum of lysine NH_3^+ groups of protein G B1 domain at pH 5.45, 278 K in 40% D_2O . Three peaks are seen for the lysines 18, 21, 36, and 58, corresponding to NH_3^+ , NH_2D^+ , and NHD_2^+ groups (bottom right to top left, respectively: see labels on lysine 18). From Ref. 5 with permission from the American Chemical Society.

From Fig. 5 as well $^1\Delta\text{N}(\text{D})$ as $^2\Delta\text{H}(\text{D})$ isotope effects can be measured. The isotope effects turned out to be very similar for both what the crystal structure had indicated could be salt bridges and those not. So the conclusion as seen in Fig. 6 is that no salt bridges is to be found in protein G B1 domain.

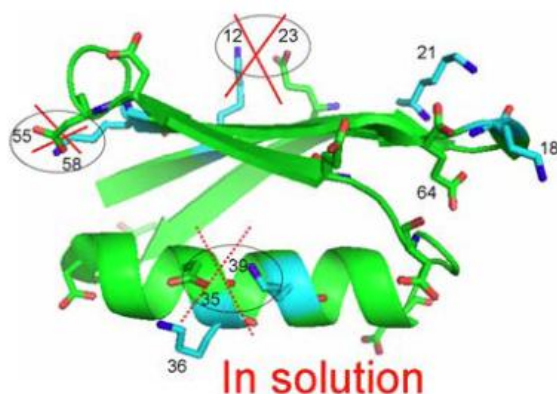


Figure 6. Crystal structure of protein G B1 domain. The three salt bridges 35-36, 55-58 and 12-23 are crossed over as they do not exist in solution. From Ref. 5 with permission from the American Chemical Society

In contrast to protein G, salt bridges could be established in barnase in solution. Lysine 27 forms a salt bridge to Aspartic acid 54. Furthermore, if Arg 69 is mutated to Lys69, the latter also forms a salt bridge. Especially $^2\Delta\text{H}(\text{D})$ is a good indicator as this becomes more negative when forming a salt bridge. In the absence of a salt bridge $^1\Delta\text{N}(\text{D})$, $^2\Delta\text{H}(\text{D})$: 0.363 \pm 0.004 ppm, -0.026 \pm 0.003 ppm, whereas in the presence of a salt bridge: 0.352 \pm 0.004 ppm, -0.033 \pm 0.002 ppm. ^[13]

Salt bridges may also involve arginines. Isotope effects were measured in the L99 mutant of T4 lysozyme. ^[14]

Iwahara et al. ^[12] demonstrated a salt bridge between lysine side-chains and the phosphate groups of DNA in the homeodomain-DNA complex also observing a $^1\Delta\text{N}(\text{D})$.

5.1. Theoretical calculation of isotope effects on chemical shifts

Theoretical calculations of isotope effects on chemical shifts can be based on the Jameson approach. ^[15] For ammonium ions this is shown in Table 1. The isotope effects on chemical shifts are calculated by shortening the NH bond length mimicking a ND bond.

Table 3. Calculated ^{15}N nuclear shieldings,* $^1\Delta\text{N}(\text{D})$, $^2\Delta\text{H}(\text{D})$ and NH nuclear shieldings of hydrogen-bonded protons.

$R_{\text{N}\cdots\text{N}}$	δN	$^1\Delta\text{N}(\text{D})$	$^1\Delta\text{N}(\text{D})^d$	δNH^e	$^2\Delta\text{H}(\text{D})$	$^2\Delta\text{H}(\text{D})^d$
2.62	231.6	0.31	—	15.1	-0.025	—
2.72	233.4	0.3	0.37	17.1	-0.0183	-0.012
3.02	237.4	0.44	0.48	21.1	-0.010	-0.003
3.2	240.5	0.52(5)	0.56	23.9	0	0.005
4.02	241.4	0.58	0.60	24.5	0.003	0.005
3.02 ^a	237.4	0.7	—	21.1	0.01 ^a	—

*Absolute shieldings. $R(\text{N}_1\cdots\text{H}_2) = 1.019 \text{ \AA}$. ^b $R(\text{N}_1\cdots\text{H}_3) = 1.019 \text{ \AA}$. ^cHydrogen part of hydrogen bond (Fig. 7). ^d $\text{NH}_3\text{D}\cdots\text{H}_2\text{O}$ complex. Data from Ref. 26. ^e $^2\text{H}_3(\text{D})$ is given. $^2\Delta\text{H}_2(\text{D}) = 0$.

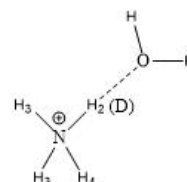


Table 1. $^1\Delta\text{N}(\text{D})$ and $^2\Delta\text{H}(\text{D})$ of deuteriated ammonium ion complexed either with ammonia or water molecule (figure). Taken from Ref. 8 with permission from the Danish Chemical Society.

From Table 1 it is seen that $^1\Delta\text{N}(\text{D})$ decreases as either ammonia or water is moving closer to the ammonium and for $^2\Delta\text{H}(\text{D})$ even a change in sign is seen. This approach has also been used for protein G B1 domain. [5] A more recent approach is to calculate isotope effects by a direct treatment of the H/D effect using a multicomponent ab initio molecular orbital method. [16]

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