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NMR Determination of Protein pK_a Values in the Solid State

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Abstract

Charged residues play an important role in defining key mechanistic features in many biomolecules. Determining the pK_a values of large, membrane or fibrillar proteins can be challenging with traditional methods. In this study we show how solid-state NMR is used to monitor chemical shift changes during a pH titration for the small soluble β1 immunoglobulin binding domain of protein G. The chemical shifts of all the amino acids with charged side-chains throughout the uniformly-¹³C, ¹⁵N-labeled protein were monitored over several samples varying in pH; pK_a values were determined from these shifts for E27, D36, and E42, and the bounds for the pK_a of other acidic side-chain resonances were determined. Additionally, this study shows how the calculated pK_a values give insights into the crystal packing of the protein.

Keywords

Chemical shift perturbation; GB1; Magic-angle spinning; pK_a determination; Solid-state NMR

Charged residues of a protein play an important role in catalytic reactions, protein stability and substrate binding.^{1–3} Many enzymatic reactions are controlled by the reversible ionization of charged residues within the active site. This ionization is determined by the intrinsic pK_a, which for residues involved in enzymatic mechanisms can deviate greatly from canonical values.^{1,4–6} Moreover, pK_a values are related to the pH range over which proteins are stable, consistent with the observation of pK_a differences among the unfolded and folded proteins,^{2,7} with aberrant pK_a values often observed in the context of hydrogen bonding, through coupled protonation and deprotonation events.⁸ Knowledge of pK_a values is useful for purposes of optimizing the absorption and stability of drugs, as well as for identifying substrate interactions in a binding pocket. The central role of ionization events in enzymology has driven the biophysical chemical community to develop methods to accurately determine pK_a values across a variety of platforms.

Solution NMR and infrared (IR) spectroscopy monitor changes in spectroscopic signatures as a function of pH to determine pK_a values.⁹ With NMR, individual protonation states can be monitored throughout the titration by tracking chemical shift changes for each resonance to yield mechanistic information¹⁰ and pH dependent protein stability.² However, large and nonsoluble systems, such as membrane proteins, fibrils and other large crystallized proteins,

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Supporting Information Available: Details of the data acquisition; full bandwidth spectra at several pH values; sample preparation details; data fitting parameters; backbone chemical shift assignments at each pH value; additional chemical shift versus pH value plot of Lys and other amino acids. This material is available free of charge via the Internet at <http://pubs.acs.org>.

are not typically amenable to these methods. For both protein fibrils and membrane proteins, X-ray structures are rare or not available to obtain pK_a values from calculations either. When structures do exist, the accuracy of the calculated pK_a is limited by the ability to correctly determine side-chain conformations and ionization states of nearby charged residues making moderate resolution crystal structures non-ideal candidates for this method.^{11,12} The importance of these systems in disease, cell signaling and ion transfer events creates a need for an alternative method for studying electrostatic interactions.

With solid-state NMR (SSNMR), large and insoluble systems can be examined to provide detailed chemical bonding and environmental information. For example, in the transition state analogue (TSA) inhibitor, carboxymethyldehia coenzyme A (CMX) bound to citrate synthase SSNMR was used to measure anisotropic chemical shifts of carboxyl groups to determine protonation states and geometry of hydrogen bonds.¹³ Additionally, isotropic chemical shifts were utilized to study rhodopsin and bathorhodopsin and the chromophore retinylidene to gain insights into electrostatic interactions and protonation states in ligation.^{14–18} However, these previous SSNMR studies were completed for a small selectively labeled cofactor in the protein systems using one- and two-dimensional experiments, and as such would require many samples in order to assess protonation states of several sites in the protein. Due to the complexity of these issues, no full titration curve to obtain exact pK_a values, as routinely done now in solution NMR, has yet been completed utilizing high-resolution SSNMR. In this work, we demonstrate that SSNMR can determine both specific pK_a values and set bounds for multiple acidic residues in a uniformly labeled protein using multidimensional homo and heteronuclear experiments. The pK_a values determined in this work give insight of the protein stability and packing of $\beta 1$ immunoglobulin binding domain of protein G (GB1) in a nanocrystal environment providing new and complimentary data to pK_a values determined in solution. Thus, for soluble proteins which can be studied by both solution and solid-state NMR the determination of pK_a values using both methods would provide exciting new details related to protein stability, crystal contacts, and salt bridges, which could in turn examine differences between the solution and solid or crystalline structures. Additionally, the methods developed within this study can be extended to larger soluble and membrane systems, such as transmembrane ion pumps and fibrillar proteins, which are uniquely suited to SSNMR studies.^{19–24}

Seven crystalline slurries of uniformly-¹³C,¹⁵N labeled GB1 were prepared using pH controlled protein buffer and precipitate solutions ranging from pH 2.42 to 5.68. Crystalline material from each slurry was isolated by centrifugation and packed into a SSNMR rotor with residual mother liquor to allow proton exchange with free carboxyl groups. The complete backbone and side-chain chemical shifts were identified for every charged residue in GB1, Asp, Glu and Lys, and Asn and Gln at every titration point. ¹³C-¹³C, ¹⁵N-(¹³CO)-¹³CX and ¹⁵N-(¹³CA)-¹³CX two-dimensional dipolar correlation spectra were acquired on each sample and compared with previously published assignments for GB1 in the solid-state.²⁵ In cases when labeled protein can be produced in large quantities, such as GB1, individual samples can be prepared at each pH value to accelerate sample preparation and data collection efforts. The methods presented in this work can be applied to larger proteins, such as membrane and fibrillar proteins where large quantities of labeled material are unavailable, by preparing a single sample at a single pH value and reconstituting the material after data acquisition and varying the pH.

The GB1 samples prepared at pH 2.53 and 2.83 were found to be unfolded with the chemical shifts predominantly consistent with a random coil secondary structure, as well as some Thr residues with secondary chemical shifts consistent with beta-sheet formation. Solution NMR studies reported that GB1 retained its structure to pH values as low as 1.8 in high and low salt solutions.^{7,8,26,27} The different behavior of the solid preparation reports on

the energy the lattice interactions contribute to stabilizing the crystalline protein structure. The protonation of the acidic residues at low pH values, disturbs the crystal packing interactions, likely disrupting salt bridges and frustrating the formation of the crystal lattice.

For samples prepared at $\text{pH} > 3.6$, the protein retained its structure such that the backbone ^{13}C resonances are conserved throughout the entire pH range for the hydrophobic residues; Val, Ile, Ala and Leu retain similar shifts in the ^{13}C - ^{13}C spectra for samples both at the highest (5.64) and the lowest (3.63) pH values (Figure 1). The charged residues (Asp and Glu) and polar residues (Asn, Gln and Thr), display small CA-CB shift differences at the varying sample pH values (Figure 1). Small chemical shift perturbations are also observed for a small subset of the CA-C' correlations through this pH range (Figure 1). In contrast, much larger resonance changes were observed for the charged residues. For example, the chemical shifts for the Asp and Glu amino acids were shifted upfield by ~ 0.5 to ~ 3 ppm as the pH was decreased, consistent with partial to full protonation of these residues.²⁸ The largest chemical shift differences over this pH range were observed for the acidic residues, with D46, D36, E15, E42, E27 and M1 displaying significant CA-CB perturbations due to their proximity to a titrating carboxyl group.²⁷

Thus samples prepared at $\text{pH} = 5.22$ and 5.64 were in very good agreement and could be assigned from the chemical shift assignments published by Franks *et al.*²⁵ *De novo* assignments were completed for the $\text{pH} = 3.63$ sample using backbone walk procedures utilizing NCACX and NCOCX three dimensional data sets.²⁹ This assignment procedure is very well established and has been used to assign several large soluble and membrane proteins.^{25,29-40} The $\text{pH} = 3.63$ assignments were used to identify resonances for the acidic amino acids in $\text{pH} = 3.89$ and 4.53 samples. The complete backbone and side-chain chemical shifts were identified for every Asp, Asn, Glu, and Gln residue in GB1 at every titration point. Chemical shift assignments were confirmed in a minimum of three spectra within a standard deviation of ± 0.10 ppm (BMRB accession number 16873).

The acidic side-chain carbonyl shifts remained relatively constant at a pH of 5.64, 5.22 and 4.55 (Figure 2). At $\text{pH} = 3.95$, all five Glu and D36 shifted upfield, due to protonation. The average determined side-chain pK_a in a protein for Glu is 4.1 ± 1.0 , and for Asp it is lower at 3.1 ± 1.0 in basic proteins to 3.9 ± 0.9 in acidic proteins.²⁸ Since GB1 has a pI of 4.2, we expect that the majority of Glu residues and approximately half of the Asp residues should titrate in the pH range sampled. Our results show that only one Asp, D36, has side-chain carbonyl chemical shifts consistent with a protonated acid at $\text{pH} = 3.63$ (Figure 2); the other residues did not titrate, displaying only slightly shifted CG resonances over the pH range.

The complete chemical shifts for two acidic residues (D40 and E56) could not be assigned at the low pH values, $\text{pH} = 3.63$ and 3.95 , since the signals from these residues were broad and weak. The D40 CB and CG resonances were not observed at $\text{pH} = 3.63$ and $\text{pH} = 3.95$, and the N-CA correlations were weak ($\text{SNR} = 7$); therefore the pK_a of D40 could not be determined in this study. Two E56 CG-CD correlations were observed in the ^{13}C - ^{13}C SPC5 mixing spectrum at $\text{pH} = 3.63$, which suggest chemical exchange or inhomogeneity at this site, and therefore its pK_a was also not determined. These observations may be consistent with proton exchange at the intermediate timescale (μs to ms), which would most likely occur at pH values close to the pK_a .

pK_a values were determined for acidic residues that experience complete protonation over the pH range sampled. Figure 2 shows the observed chemical CG/CD chemical shifts for the Asp and Glu of GB1 plotted as a function of sample buffer pH. D36, E27 and E42 all experienced complete protonation and quantitative pK_a values were determined. Although the E19 and E15 CD resonances had shifted upfield by ~ 1.5 ppm from $\text{pH} = 5.64$ to $\text{pH} =$

3.63, the fully protonated chemical shift could not be confirmed, thus pK_a values could not be quantitatively determined. However, an upper pK_a bound of 4.0 pH units can be inferred from the pH range sampled. The residues, C-terminus D22, D46, D47 and E56, which did not display significant chemical shift deviations, can be assumed to have pK_a values below 3.63.

The calculated pK_a values for D36, E42 and E27 as well as the bounding values for other E and D residues are given in Table 1. The pH titration curves of D36, E42, and E27 have a sigmoidal shape, typical for a single titrating residue, and were fit to a modified Henderson-Hasselbalch equation. The protonated chemical shift and deprotonated chemical shifts were obtained from the pH = 3.63 and 5.64 samples, respectively. The fitted curves (Figure 2) show good agreement with the experimental data when the pK_a values and the Hill coefficient are simultaneously varied during the fitting. The solid-state E42 and E27 pK_a values are similar to the expected value for Glu residues ($pK_a=4.2$).²⁸ The D36 determined pK_a is ~0.4 pH units higher than the expected value ($pK_a=3.9$) for an acidic protein.

pK_a perturbations of residues can be caused by several electrostatic interactions, such as charge-charge interactions, hydrogen bonding and desolvation.^{1,28} In the case of solvent-exposed D36 CG, the most likely of these explanations is that a nearby negative charge shifts the pK_a value up, discussed further below. The Hill coefficients, which report on cooperativity of the protonation event with other local charges, were determined for E27, D36 and E42 and they ranged from 3.1 to 4.5 units (Table 1). These values are three to four-fold larger than those typically observed for solvent exposed residues from solution NMR measurements. Hill coefficients on the order observed in our solid-state NMR measurements are the result of a highly coupled protonation event, which we attribute to the propagation of structural changes throughout the highly concentrated environment of the crystal lattice.

Table 1 also shows the pK_a comparisons between the SSNMR determined values for E27, D36 and E42, as well as the other E and D bounded pK_a values, and the pK_a values determined by other methods. The SSNMR pK_a values are in very good agreement with both those determined for wild type GB1 by solution NMR, as well as by the MCCE calculation.^{8,27,41} There are small differences between the SSNMR and calculated E27, D36 and E42 values. Both methods examined the trigonal crystal packing arrangement of GB1,^{42,43} thus similar packing interactions would be present and observed differences could be from the uncertainties of both methods. The strongest agreement was observed for the D36 pK_a between SSNMR and that determined by ¹³C solution NMR for the QQD-GB1 (T2Q, N8D, N37D) mutant.^{7,26,27} The addition of a positive charge near D36 shifts the pK_a up in the QQD-GB1 mutant.²⁷ A similar event could be occurring in the SSNMR preparation from the protein crystal packing. This was confirmed in the crystal structure (2QMT), with D36 packed close to itself in the crystal lattice (Figure 3).

The three titrating residues (E27, D36 and E42), although solvent-exposed, were not involved in salt bridges or hydrogen bonding according to the X-ray crystal structure (Figure 3).⁴² Three of the acidic residues that did not titrate above a pH of 3.63, E15, D47 and E56, were involved in a salt bridge with a nearby lysine.⁴² For example, from the GB1 structure (PDB ID: 2QMT) it can be determined that E15 is involved in an intramolecular salt bridge with K4 (3.05 Å), as D47 is in a salt bridge with K50 (3.27 Å) and E56 with K10 (3.39 Å). Additionally, by recreating the crystal packing E27 is shown to be 3.81 Å from K31, E42 is 4.81 Å from a K4 intermolecular contact, and D40 is 4.95 Å from an intermolecular contact with K10. During the titration the Lys N ζ resonances essential have no deviation of their chemical shift (shown in the SI), indicating that the salt bridges are intact during the titration. As the pH is lowered below ~3, these salt bridges and other hydrogen bonds, which are critical for protein stability and crystal packing, are broken, denaturing the protein.

In this work we have determined specific pK_a values for acidic residues, E27, D36 and E42, in the nanocrystalline protein GB1 by SSNMR. This demonstrates the ability of SSNMR to study electrostatic events and determine pK_a values. The determined pK_a values were in good agreement with those previously determined by solution NMR,⁸ with one residue demonstrating a shifted pK_a due to crystal packing in the solid state that was not observed in solution. These SSNMR methods for pK_a determination can now be extended to large crystalline, fibrillar, and membrane proteins that other methods have traditionally experienced challenges and use this information to elucidate key mechanistic, drug targeting and interaction, and folding and unfolding information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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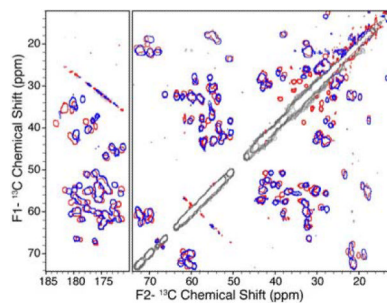


Figure 1. Overlay of ^{13}C - ^{13}C 2D spectra acquired on uniformly- ^{13}C , ^{15}N labeled GB1 at pH = 3.63 (blue) and pH = 5.64 (red) using SPC5₃ mixing.

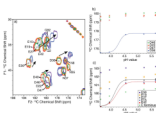


Figure 2. (a) Side-chain carbonyl chemical shifts over the pH titrated range. Expansion of the CG-CO and CD-CO region of the ^{13}C - ^{13}C 2D spectra acquired with SPC5₃ mixing for samples at pH=3.63 (purple), pH=3.95 (blue), pH=4.55 (green), pH=5.22 (orange) and pH=5.64 (red), and pH dependence of ^{13}C side-chain carbonyl chemical shifts in nanocrystalline GB1. (b) Aspartic acid CG resonances and (c) glutamic acid CD and C-terminus carbonyl resonances plotted against sample buffer pH showing fitted curves for D36 (blue), E27 (black) and E42 (red).

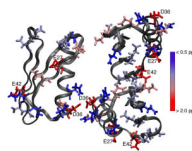


Figure 3. Three GB1 molecules in trigonal crystal packing (PDB:2QMT) with chemical shift differences between low (pH = 3.63) and high (pH = 5.64) pH samples for the carboxylic acid side-chains of Asn/Asp/Gln/Glu residues colored from no change (blue) to > 2.0 ppm chemical shift differences (red). Residues with > 2.0 ppm chemical shift differences are labeled.

Table 1

SSNMR determined pK_a values for nanocrystalline GB1 compared to values determined by solution NMR experiments and calculations from X-ray structure.

	^{13}C SSNMR	Hill coefficient (nH)	^1H NMR ⁸	Calculated (IPGB) ⁴¹	^{13}C NMR QO-D-GB1 ²⁷	^{13}C NMR QO-D-GB1 (0.5 M sat) ²⁷
E15	< 4.0		4.4	5.0	4.6	4.7
E19	< 4.0		3.7	4.1	3.9	4.0
D22	< 3.6		2.9	2.3	3.0	3.2
E27	4.1 ± 0.1	3.8 ± 0.6	4.5	4.9	4.8	4.9
D36	4.3 ± 0.1	4.5 ± 0.7	3.8	4.9	4.2	4.1
D40	N.D.		4.0	4.4	4.1	4.1
E42	4.2 ± 0.2	3.1 ± 0.6	4.4	4.0	4.9	4.6
D46	< 3.6		3.6	3.8	3.8	3.9
D47	< 3.6		3.4	3.3	3.1	3.4
E56	< 3.6		4.0	3.7	3.8	3.9
C terminus	< 3.6				3.1	3.2