Published in final edited form as:

Surf Sci. 2010 August 15; 604(15-16): L39–L42. doi:10.1016/j.susc.2010.02.026.

The Role of Basic Amino Acids in the Molecular Recognition of Hydroxyapatite by Statherin using Solid State NMR

Moise Ndao¹, Jason T. Ash^{1,*}, Patrick S. Stayton², and Gary P. Drobny¹

¹Department of Chemistry, University of Washington, Seattle, Washington 98195.

²Department of Bioengineering, University of Washington, Seattle, Washington 98195.

Abstract

Organisms use proteins such as statherin to control the growth of hydroxyapatite (HAP), which is the principal component of teeth and bone. Though much emphasis has been placed on the acidic character of these proteins, the role of their basic amino acids is not well understood. In this work, solid state nuclear magnetic resonance was used to probe the interaction of the basic arginine side chains with the HAP surface. Statherin samples were individually labeled at each arginine site, and the distance to the surface was measured using the Rotational Echo DOuble Resonance (REDOR) technique. The results indicate a strong coupling between the R9 and R10 residues and the phosphorus atoms on the surface, with internuclear distances of $4.62 \pm 0.29 \text{ \AA}$ and $4.53 \pm 0.16 \text{ \AA}$, respectively. Conversely, results also indicate weak coupling between R13 and the surface, suggesting this residue is more removed from the surface than R9 and R10. Combining these results with previous data, a new model for the molecular recognition of HAP by statherin is constructed.

Organisms are able to carefully control the growth of mineral phases such as silica, calcite, and hydroxyapatite (HAP) [1–3] through the use of proteins at the organic-inorganic interface [4–8]. Understanding of the structure and dynamics of these peptides, particularly on the inorganic surface, is crucial to understanding their function. Additionally, this information may improve the compatibility of biomaterials. Though these systems are difficult to probe using diffraction techniques, the surface coverages are often large enough to utilize solid state nuclear magnetic resonance (SSNMR) spectroscopy.

One such protein is statherin, a 43-residue acidic phosphopeptide present in saliva [9–11]. Statherin binds calcium ions in solution and adsorbs onto HAP surfaces [12–14]. Statherin and related proteins inhibit both precipitation and secondary crystal growth [15,16]. They help maintain supersaturation of calcium and phosphate ions in saliva and act as a boundary lubricant [17–21]. The primary sequence of statherin is: D¹pSpSEEKFLRRIGRFGYGYGPYQPVPEQPLYLQPYQPQYQQYTF⁴³, where pS represents phosphorylated serine residues. The first five amino acids in the N-terminus of statherin, comprising one aspartic acid, two phosphorylated serines, and two glutamic acid residues, are known to be important in the adsorption to HAP [22]. Polyacidic regions are

© 2010 Elsevier B.V. All rights reserved.

*Corresponding author. jason.jash@gmail.com, Department of Chemistry, University of Washington, Box 351700, Seattle, WA 98195-1700.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

common in proteins that interact with inorganic surfaces [23]. The acidic domains mediate protein binding to hydroxyapatite, and a number of models have been proposed [24,25].

Though basic synthetic polymers such as poly-L-lysine are known to adsorb onto HAP surfaces [26], the role of basic amino acids in biomineralization is not well understood. Statherin contains four basic residues: lysine (K) at residue 6 and arginines (R) at residues 9, 10, and 13. Thermodynamic measurements have shown that mutation of these basic residues to alanine significantly decreases the equilibrium constant for adsorption [27], while computational structure prediction methods suggest that the four basic residues of statherin form a motif that is critical for adsorption [28,29]. Although SSNMR measurements have shown that the side chain of K6 lies in close proximity of the surface [31], the role of the three arginine residues remains unclear.

To address this issue, uniformly ^{13}C labeled Fmoc-Arg(Pbf)-OH was purchased from Isotec (Sigma-Aldrich) and a third generation Rainin PS3 solid phase peptide synthesizer was used to synthesize a total of three statherin samples, one labeled at residue 9 (stR9), one at residue 10 (stR10), and one at residue 13 (stR13). Following HPLC purification, all samples were dissolved in PBS buffer and bound to 100 mg of HAP seed crystals with a surface area of $57\text{ m}^2/\text{g}$ that were provided by A. Campbell (Battelle Pacific Northwest National Laboratory, Richland, WA). Typically, 12 mg of statherin adsorbed onto the surface. The resulting samples were left hydrated in PBS buffer and approximately 70 mg of the hydrated material, estimated to be 50 % solvent, were packed into NMR rotors. The samples are hereafter designated stR9hap, stR10hap, and stR13hap, respectively. More details of the sample preparation may be found elsewhere [30].

Once synthesized, the interaction of the arginine residues in statherin with the HAP surface was explored by utilizing ^{13}C Cross Polarization Magic Angle Spinning (CPMAS) and $^{13}\text{C}\{^{31}\text{P}\}$ Rotational Echo Double Resonance (REDOR) NMR [32], whose pulse sequences are outlined in Figure 1 (a) and (b), respectively. The REDOR experiment is used here to measure the dipolar coupling, and hence the distance, between the ^{13}C labeled arginine in the protein and the phosphorus atoms on the HAP surface. The SSNMR experiments were performed on a home-built spectrometer attached to a 11.74 T superconducting magnet (500.11 MHz ^1H frequency). Experiments were performed using a 4-mm HXY triple-resonance Varian/Chemagnetics magic angle spinning probe under a sample spinning speed of $6000 \pm 1\text{ Hz}$. As illustrated in Figure 1 (b), an XY8-phase cycling $^{13}\text{C}\{^{31}\text{P}\}$ REDOR with alternating π -pulses on both the ^{13}C -observed and ^{31}P -dephasing channel was used. Experiments were performed at -40°C to improve cross-polarization efficiency and to avoid sample degradation during the long acquisition periods. More details can be found elsewhere [30]. The resulting experimental REDOR curves were fit using the simulation program SIMPSON [33–36]. Simulations were performed using the direct algorithm, along with a set of 678 Euler angles, specified by Conroy-Wolfsberg [37,38] scheme and 10 γ angles. Though homonuclear ^{13}C - ^{13}C couplings are present, initial simulations of both the REDOR dephasing and reference spectra indicated that their inclusion in the simulations would have only a small overall effect ($< 0.05\text{ \AA}$) on calculated distances. These effects were therefore ignored. Homonuclear ^{31}P - ^{31}P couplings arising from the network of coupled ^{31}P spins on the HAP surface were simulated using a reduced dephasing system composed of two spins with a large effective dipolar coupling of 600 Hz. Simulations show that heteronuclear dipolar couplings to the two nearest neighbor ^{31}P spins in ^{15}N labeled diammonium hydrogen phosphate can be accurately reproduced in this fashion [39,40]. Therefore, the data were fit using a 3-spin triad (CPP) model between the C_α and two phosphorus spins from the HAP surface, as diagrammed in Figure 1(c). In the simulations, both ^{13}C - ^{31}P couplings were varied while keeping the ^{31}P - ^{31}P coupling

constant fixed at 600 Hz. This approach has been used to determine distances from the HAP surface to the sidechains of both K6 [31] and E5 [30].

Figure 2 shows the ^{13}C cross polarization magic angle spinning (CPMAS) spectra of stR9hap, stR10hap, and stR13hap, referenced relative to TMS. Also displayed are vertical lines indicating resonance positions of the FMOC-Arg(Pbf)-OH starting material along with spectral assignments. In previous work on this system, the interaction of ^{13}C nuclei in the aromatic ring of F14 with the HAP surface induced a 2-ppm change in chemical shift [41]. However, the present samples show that all carbon resonances, including the C_ζ possibly near the surface, are not shifted relative to the starting material within the experimental linewidths (~ 2 ppm for C_ζ , 4 ppm for $\text{C}_{\alpha,\beta}$, and 7 ppm for $\text{CO}, \text{C}_{\beta,\gamma}$). This insensitivity of the chemical shift of C_ζ to the surface is not surprising given that they are also rather insensitive to structural variation. For example, the structures compiled in the BioMagResBank [42] show a C_ζ chemical shift distribution of approximately 0.5 ppm (FWHM) which is well within the observed linewidth. More importantly, structural models predict that the terminal nitrogens of the arginine, and not C_ζ , interact directly with the surface [29]. Though a 2-ppm shift was noted for the terminal ^{15}N of K6 in a statherin fragment upon binding to HAP, the molar surface coverage of full length statherin is five times smaller than that of the fragment, making such measurement less feasible [22].

It is important to note that the insensitivity of the ^{13}C chemical shifts to the surface is not necessarily indicative of their proximity to the surface. That information is more reliably deduced from the REDOR measurements for stR9hap, stR10hap, and stR13hap, which are shown in Figure 3. These data were obtained by integration of the C_ζ region of the ^{13}C spectrum for each sample, as this was the only resonance exhibiting significant dephasing. Because the ^{31}P spins in the side chain phosphate groups of pS2 and pS3 are more than 9 Å from any of the carbon labels, the dephasing observed for stR9hap and stR10hap are indicative of dipolar interactions between the arginine C_ζ and the ^{31}P spins within the biomineral HAP surface. Qualitatively, this result is interesting because previous measurement have shown that K6 also interacts with the surface [31]. Since the predicted α -helical structure of the N terminus of statherin requires 3.6 residues per helical turn, both R9 and R10 are expected to be on the same side of the peptide as is K6, allowing their side chains to interact with the surface as well. These results also indicate that two adjacent basic residues interact with the surface. This is in contrast to previous work on the acidic side chains of statherin, where only one of the two adjacent glutamate residues interacted with the surface [30]. In addition to the qualitative interpretation described above, simulation of the REDOR data allows for a quantitative measurement of the distance between the C_ζ and the phosphorus spins on the HAP surface. A χ^2 analysis found best agreement with the experimental data when both ^{13}C - ^{31}P distances were 4.62 ± 0.29 Å for stR9hap and 4.53 ± 0.16 Å for stR10hap.

The interaction of R9 and R10 with the surface is consistent with the solution-state model of statherin, as both of these residues lie on the same side of the α -helix as K6, whose side chain has also been shown to interact with the surface [43]. In solution models, the N terminus of statherin is helical up to glycine-12, which acts as a helical cap. The close proximity of the positively charged K6, R9, and R10 residues is stabilized by the negatively charged phosphoserine and glutamate residues found at positions 2–5. Since there are no such negatively charged residues near R13, it has a propensity to orient itself away from R9 and R10.

In contrast, the adsorbed state RosettaSurface model predicts that the negatively charged phosphate groups on the surface support a conformational change in statherin, extending the α -helix conformation beyond R13 [29]. In this model, R13 lies on the same side of the

peptide as K6, R9, and R10, and interacts with the HAP surface. The four basic residues, K6, R9, R10, and R13, form hydrogen bonds with three phosphate oxygen atoms that meet on the surface, known as Interstices of Phosphate Oxygen Triad (IPOT) [28].

If this were in fact the case, the dephasing for stR13hap would be similar to that of stR9hap and stR10hap. However, the weak dephasing observed for stR13hap indicates that the distance from C γ to the surface is beyond the detectable range for ^{13}C - ^{31}P REDOR, which is around 7 Å. Though these data appear to be more consistent with the solution state structural models, it is important to point out that the K6/R9/R10/R13 IPOT model was first predicted based on adsorption to a perfect [001] face of the HAP crystal [28]. Subsequent work indicates that the frequency of R13 interactions decreases when other crystal faces are considered [29]. Thus, it is possible that the discrepancy here is due simply to a marked deviation of our HAP sample from [001] geometry [29].

With these data, a model consistent with the current SSNMR data as well as previous NMR, calorimetric, and structure prediction results for the molecular recognition of HAP by statherin has been constructed and is depicted in Figure 4. In this model, the side chains of E5, K6, R9, and R10 all lie within 4.5 Å of the surface, while those of E4, F7, and R13 are more than 7 Å away. It is important to note here that there may be additional side chain interactions with the surface that have yet to be measured experimentally. Most notable are pS2 and pS3, as dephosphorylation of these residues is known to decrease the binding affinity by a factor of almost 9 [22,44], and both solution and surface models predict these residues to be correctly oriented to interact with the surface. Further studies are underway involving not only measurements of other surface-side chain interactions in statherin, but also probing the role of protein and water dynamics at the interface.

Acknowledgments

We thank Dr. Allison Campbell and Dr. Wendy Shaw for the hydroxyapatite sample, as well as Dr. Nicholas Breen for useful discussions. This research was supported by National Institutes of Health grants RO1 DE12554-06 and RO1 GM074511-03.

References

1. Lowenstam, H.; Weiner, S. *On Biomineralization*. New York: Oxford University Press; 1989.
2. Simkiss, K.; Wilbur, K. *Biomineralization: Cell Biology and Mineral Deposition*. New York: Academic Press; 1989.
3. Weiner S, Addadi L. Acidic macromolecules of mineralized tissues - the controllers of crystal-formation. *Trends Biochem. Sci.* 1991; 16:252–256. [PubMed: 1926334]
4. Weiner S, Addadi L. Design strategies in mineralized biological materials. *J. Mater. Chem.* 1997; 7:689–702.
5. Mann S. Molecular tectonics in biomineralization and biomimetic materials chemistry. *Nature.* 1993; 365:499–505.
6. Hunter G, Goldberg H. Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. *Biochem. J.* 1994; 302:175–179. [PubMed: 7915111]
7. Hunter G, Hauschka P, Poole A, Rosenberg L, Goldberg H. Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. *Biochem. J.* 1996; 317:59–64. [PubMed: 8694787]
8. Fujisawa R, Kuboki Y. Conformation of dentin phosphophoryn adsorbed on hydroxyapatite crystals. *Eur. J. Oral Sci.* 1998; 106:249–253. [PubMed: 9541233]
9. Hay D. Isolation from human parotid saliva of a tyrosine-rich acidic peptide which exhibits high affinity for hydroxyapatite surfaces. *Arch. Oral Biol.* 1973; 18:1531–1541. [PubMed: 4522816]

10. Hay, D.; Schlesinger, D. Calcium-binding proteins and calcium function. In: Wasserman, R.; McLennan, D.; Siegel, F., editors. *Proceedings of International Symposium on Calcium-Binding Proteins and Calcium Function in Health and Disease*; New York. Elsevier Science Publishers; 1977. p. 401-408.
11. Hay D, Moreno E, Schlesinger D. Phosphoprotein-inhibitors of calcium-phosphate precipitation from salivary secretions. *Inorg. Perspect. Biol. Med.* 1979; 2:271–285.
12. Johnsson M, Levine M, Nancollas G. Hydroxyapatite binding domains in salivary proteins. *Crit. Rev. Oral Biol. Med.* 1993; 4:371–378. [PubMed: 8396998]
13. Moreno E, Kresak M, Hay D. Adsorption of 2 human parotid salivary macromolecules on hydroxyapatites, fluorhydroxyapatites and fluorapatites. *Arch. Oral Biol.* 1978; 23:525–533. [PubMed: 281196]
14. Goobes R, Goobes G, Campbell C, Stayton P. Thermodynamics of statherin adsorption onto hydroxyapatite. *Biochemistry.* 2006; 45:5576–5586. [PubMed: 16634639]
15. Proctor G, Hamdan S, Carpenter S, Wilde P. A statherin and calcium enriched layer at the air interface of human parotid saliva. *Biochem. J.* 2005; 389:111–116. [PubMed: 15769251]
16. Hay, DI.; Moreno, EC.; Tenovuo, JO., editors. *Human saliva: Clinical Chemistry and Microbiology*. Boca Raton, FL: CRC Press; chapter 5; p. 131-150.
17. Schupbach P, Oppenheim F, Lendenmann U, Lamkin M, Yao Y, Guggenheim B. Electron-microscopic demonstration of proline-rich proteins, statherin, histatins in acquired enamel pellicles in vitro. *Eur. J. Oral Sci.* 2001; 109:60–68. [PubMed: 11330936]
18. Li J, Helmerhorst E, Yao Y, Nunn M, Troxler R, Oppenheim F. Statherin is an in vivo pellicle constituent: identification and immuno-quantification. *Arch. Oral Biol.* 2004; 49:379–385. [PubMed: 15041485]
19. Vitkov L, Hannig M, Nekrashevych Y, Krautgartner W. Supramolecular pellicle precursors. *Eur. J. Oral Sci.* 2004; 112:320–325. [PubMed: 15279650]
20. Yin A, Margolis H, Yao Y, Grogan J, Oppenheim F. Multi-component adsorption model for pellicle formation: The influence of salivary proteins and non-salivary phospho proteins on the binding of histatin 5 onto hydroxyapatite. *Arch. Oral Biol.* 2006; 51:102–110. [PubMed: 16055080]
21. Douglas W, Reeh E, Ramasubbu N, Raj P, Bhandary K, Levine M. Statherin - a major boundary lubricant of human saliva. *Biochem. Biophys. Res. Commun.* 1991; 180:91–97. [PubMed: 1718282]
22. Raj P, Johnsson M, Levine M, Nancollas G. Salivary statherin - dependence on sequence, charge, hydrogen-bonding potency, and helical conformation for adsorption to hydroxyapatite and inhibition of mineralization. *J. Biol. Chem.* 1992; 267:5968–5976. [PubMed: 1313424]
23. Gorski J. Acidic phosphoproteins from bone-matrix - a structural rationalization of their role in biomineralization. *Calcif. Tissue Int.* 1992; 50:391–396. [PubMed: 1596774]
24. Boskey A. Osteopontin and related phosphorylated sialoproteins - effects on mineralization. *Ann. N. Y. Acad. Sci.* 1995; 760:249–256. [PubMed: 7785899]
25. Fujisawa R, Kuboki Y. Preferential adsorption of dentin and bone acidic proteins on the (100) face of hydroxyapatite crystals. *Biochimica et Biophysica Acta.* 1991; 1075:56–60. [PubMed: 1654109]
26. Fernandez V, Reimer J, Denn M. Magnetic-resonance studies of polypeptides adsorbed on silica and hydroxyapatite surfaces. *J. Am. Chem. Soc.* 1992; 114:9634–9642.
27. Goobes R, Goobes G, Shaw W, Drobny G, Campbell C, Stayton P. Thermodynamic roles of basic amino acids in statherin recognition of hydroxyapatite. *Biochemistry.* 2007; 46:4725–4733. [PubMed: 17391007]
28. Makrodimitris K, Masica D, Kim E, Gray J. Structure prediction of protein-solid surface interactions reveals a molecular recognition motif of statherin for hydroxyapatite. *J. Am. Chem. Soc.* 2007; 129:13713–13722. [PubMed: 17929924]
29. Masica D, Gray J. Solution- and Adsorbed-State Structural Ensembles Predicted for the Statherin-Hydroxyapatite System. *Biophysical Journal.* 2009; 96:3082–3091. [PubMed: 19383454]

30. Ndao M, Ash J, Breen N, Goobes G, Stayton P, Drobny G. A $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR Investigation of the Role of Glutamic Acid Residues in Statherin- Hydroxyapatite Recognition. *Langmuir*. 2009; 25:12136–12143. [PubMed: 19678690]
31. Gibson J, Raghunathan V, Popham J, Stayton P, Drobny G. A REDOR NMR study of a phosphorylated statherin fragment bound to hydroxyapatite crystals. *J. Am. Chem. Soc.* 2005; 127:9350–9351. [PubMed: 15984845]
32. Gullion T, Schaefer J. Rotational-echo double-resonance NMR. *J. Magn. Reson.* 1989; 81:196–200.
33. Bak M, Rasmussen J, Nielsen N. SIMPSON: A general simulation program for solid-state NMR spectroscopy. *J. Magn. Reson.* 2000; 147:296–330. [PubMed: 11097821]
34. Bak M, Schultz R, Vosegaard T, Nielsen N. Specification and visualization of anisotropic interaction tensors in polypeptides and numerical simulations in biological solid-state NMR. *J. Magn. Reson.* 2002; 154:28–45. [PubMed: 11820824]
35. Vosegaard T, Nielsen N. Towards high-resolution solid-state NMR on large uniformly N-15- and [C-13, N-15]-labeled membrane proteins in oriented lipid bilayers. *J. Biomol. NMR*. 2002; 22:225–247. [PubMed: 11991353]
36. Vosegaard T, Malmendal A, Nielsen N. The flexibility of SIMPSON and SIMMOL for numerical simulations in solid- and liquid-state NMR spectroscopy. *Chem. Monthly*. 2002; 133:1555–1574.
37. Conroy H. Molecular Schrödinger equation .8. A new method for evaluation of multidimensional integrals. *J. Chem. Phys.* 1967; 47:5307–5318.
38. Cheng V, Suzukawa H, Wolfsberg M. Investigations of a nonrandom numerical-method for multidimensional integration. *J. Chem. Phys.* 1987; 59:3992–3999.
39. Raghunathan V, Gibson J, Goobes G, Popham J, Louie E, Stayton P, Drobny G. Homonuclear and heteronuclear NMR studies of a statherin fragment bound to hydroxyapatite crystals. *J. Phys. Chem. B*. 2006; 110:9324–9332. [PubMed: 16671751]
40. Goobes G, Raghunathan V, Louie EA, Gibson JM, Olsen GL, Drobny GP. A REDOR study of diammonium hydrogen phosphate: A model for distance measurements from adsorbed molecules to surfaces. *Solid State Nuclear Magnetic Resonance*. 2006; 29:242–250. [PubMed: 16257188]
41. Gibson J, Popham J, Raghunathan V, Stayton P, Drobny G. A solid-state NMR study of the dynamics and interactions of phenylalanine rings in a statherin fragment bound to hydroxyapatite crystals. *J. Am. Chem. Soc.* 2006; 128:5364–5370. [PubMed: 16620107]
42. Ulrich E, Akutsu H, Doreleijers J, Harano Y, Ioannidis Y, Lin J, Livny M, Mading S, Maziuk D, Miller Z, Nakatani E, Schulte C, Tolmie D, Wenger R, Yao H, Markley J. BioMagResBank. *Nucleic Acids Res.* 2007; 36:D402–D408. [PubMed: 17984079]
43. Goobes G, Goobes R, Schueler-Furman O, Baker D, Stayton P, Drobny G. Folding of the C-terminal bacterial binding domain in statherin upon adsorption onto hydroxyapatite crystals. *Proceedings of the National Academy of Sciences*. 2006; 103:16083–16088.
44. Wikel K, Burke E, Perich J, Reynolds E, Nancollas G. Hydroxyapatite mineralization and demineralization in the presence of synthetic phosphorylated pentapeptides. *Arch. Oral Biol.* 1994; 39:715–721. [PubMed: 7980121]

Figure 1.

(a) ^{13}C CPMAS pulse sequence. (b) XY8 phase cycling $^{13}\text{C}\{^{31}\text{P}\}$ REDOR pulse sequence with alternating π pulses on both channels. (c) CPP triad model used to simulate the data, with arrows indicating the couplings that were varied in the simulations.

Figure 2.

CPMAS spectra of stR9HAP, stR10, and stR13hap acquired at ambient temperature and a spinning rate of 6 kHz along with spectral assignments. Dashed lines correspond to the positions of the labeled FMOC arginine used as the starting material for labeling of all statherin samples. For each spectrum, 50,000 transients were acquired at a repetition rate of 2 s (total acquisition time: 28 hours per sample).

Figure 3. REDOR decay plots for stR9hap, stR10hap, and stR13hap, along with spin triad (CPP) simulations. For the simulations, the ^{31}P - ^{31}P coupling was fixed at 600 Hz, while the two ^{13}C - ^{31}P couplings were varied simultaneously. Between 60,000 and 120,000 transients were acquired at for both the S and S_0 spectra of each data point. The repetition rate was 2 s (total acquisition time: 16 days per sample).

Figure 4.
Model of statherin on the HAP surface, combining the new distance constraints outlined in this work with those obtained previously.