Biosilica-Entrapped Enzymes Studied by Using Dynamic Nuclear-Polarization-Enhanced High-Field NMR Spectroscopy

Citation: Ravera, Enrico et al. "Biosilica-Entrapped Enzymes Studied by Using Dynamic Nuclear-Polarization-Enhanced High-Field NMR Spectroscopy." ChemPhysChem 16, 13 (August 2015): 2751–2754 © 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

As Published: http://dx.doi.org/10.1002/CPHC.201500549

Publisher: Wiley Blackwell

Persistent URL: http://hdl.handle.net/1721.1/113315

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

HHS Public Access

Author manuscript

Chemphyschem. Author manuscript; available in PMC 2017 February 12.

Biosilica-Entrapped Enzymes can be studied by DNP-enhanced high-field NMR

Dr. Enrico Ravera[1,‡], **Dr. Vladimir K. Michaelis**[2,‡], **Dr. Ta-Chung Ong**[2,3], **Mr. Eric G. Keeler**[2], **Dr. Tommaso Martelli**[1,4], **Dr. Marco Fragai**[1], **Prof. Robert G. Griffin**[2,*], and **Prof. Claudio Luchinat**[1,*]

[1]Magnetic Resonance Center (CERM) and Department of Chemistry "Ugo Schiff", University of Florence, 50019 Sesto Fiorentino (FI), Italy

[2]Francis Bitter Magnet Laboratory and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA, 02139, USA

Abstract

Enzymes are used as environmentally friendly catalysts in many industrial applications, and are frequently immobilized in a matrix to improve their chemical stability for long-term storage, and reusability. Recently, it was shown that an atomic-level description of proteins immobilized in a biosilica matrix can be attained by examining their magic angle spinning (MAS) NMR spectra. However, even though MAS NMR is an excellent tool for determining structure, it is severely hampered by sensitivity. In this work we provide the proof of principle that NMR-characterization of biosilica-entrapped enzymes could be assisted by high-field dynamic nuclear polarization (DNP).

Keywords

biosilica; enzyme immobilization; DNP; biomaterial; solid-state NMR

Communication

Enzymes are highly efficient catalysts that perform complex chemical reactions under mild conditions, and are ideal for reducing the environmental footprint of chemical processes.[1] Methods to immobilize enzymes in a solid matrix or support have been proposed[2] to achieve both the stabilization of the enzymes and the creation of heterogeneous catalysts. Among immobilization methods, biosilicification is a remarkable strategy inspired by nature:[3–5] polycationic molecules are used to direct and promote silicic acid polycondensation reactions[4,6], which proceed under benign conditions[7], within a few minutes[6] and with high immobilization yields.[4]

*Corresponding Authors, rgg@mit.edu; claudioluchinat@cerm.unifi.it.
[3]Present address: Department of Chemistry, Laboratory of Inorganic Chemistry, ETH-Zürich, CH-8093 Zürich, Switzerland
[4]Present address: Giotto Biotech Srl, Via Madonna del Piano 6, 50019 Sesto Fiorentino (FI), Italy
‡These authors contributed equally.

Biomaterials of such kind have attracted significant interest,[8] and their usual characterization is based on the analysis of the catalytic activity, (Figure S1). Structurally, they are difficult to characterize by standard structural biology tools such as X-ray crystallography and solution NMR, but they can be investigated by MAS NMR, which has been used to study protein-mineral interfaces[9] and the mechanistic aspects of biosilica formation by peptides from *Cylindrotheca fusiformis*[10] or by *Thalassiosira pseudonana* cells.[11] We have demonstrated that it is possible to achieve high-resolution MAS characterization of biosilica immobilized enzymes,[12] providing two examples: the catalytic domain of matrix metalloproteinase 12, in its complex with NNGH (N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid) (catMMP12)[13] and the Cu(II)-Zn(II) loaded C6A,C111S mutant of human superoxide dismutase 1 (AS-SOD).[14] Both proteins displayed highly resolved NMR spectra in the immobilized form and it was possible to ascertain that the structure of the protein was maintained.[12] However sensitivity is a persistent problem in the NMR studies of biosilica structures and bioinspired materials in general[11].

To address the challenges imposed by low sensitivity, dynamic nuclear polarization (DNP) may be applied. In DNP, the large electron polarization of a paramagnetic species is transferred to neighbouring nuclei via microwave irradiation. This efficiency of this process is optimal at cryogenic temperatures where electron spin and nuclear relaxation times increase and allow for efficient polarization transfer. Bis-nitroxide biradicals are the most successful polarizing agents due to the increased electron-electron coupling (20–35 MHz) that is required for cross effect (CE) efficiency.[15] To date solid-state DNP research faces two main issues: a) determining the kind of samples that are amenable for DNP and b) understanding and preventing the loss of resolution at cryogenic temperatures. Although several studies have demonstrated the possibility of polarizing the surface of silica-based materials by DNP,[16] it is not trivial to deduce that the method is also suited for increasing the sensitivity in the study of proteins encapsulated within a biosilica matrix. In terms of sample preparation, the protein and the radical may reside in different phases, separated by the silica matrix, as opposed to the case of a homogenous glassy preparation,[17] and as such it could give no enhancement at all, as in the case of frozen solution without cryoprotectant. On the contrary, in this work we could demonstrate that substantial enhancements are achieved in NMR sensitivity of catMMP12 and AS-SOD proteins entrapped in biosilica by employing high field DNP (5 T and 16.4 T), providing the proof of principle that high-field DNP enhancement can be obtained for these systems.

DNP-enhanced NMR spectra of biosilica-entrapped catMMP12 and AS-SOD were obtained at 5 T (211 MHz/140 GHz). The biosilica samples were treated with a cryoprotectant (60:30:10 glycerol-d_8/D_2O/H_2O)[18] containing a 10 mM concentration of the biradical polarizing agent TOTAPOL [1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)-propan-2-ol].[19] Proton DNP enhancements (ε) of 32 and 50 were achieved for catMMP12 and AS-SOD, respectively.

The presence of silica may affect the DNP properties of the sample, since it may act as a shell protecting the enzyme, and it can have non-specific interactions with the biradical.[20] Therefore we tested if the biosilica matrix could act similarly to surfactant-containing

systems[21,22] or other systems where the biradical is protected from segregation upon solvent freezing:[23–28] *i.e.*, if the use of a cryoprotectant could be avoided. For the cryoprotectant-free approach two samples were prepared by washing the solid with a 10 mM TOTAPOL solution in D_2O. DNP ε of 20 and 55 were obtained for catMMP12 and AS-SOD (Figure S2): for AS-SOD the efficiency of DNP was comparable with and without cryoprotectant, while for catMMP12 the non-cryoprotected sample yielded somewhat lower but still significant enhancement. These results suggests that the DNP efficiency depends on the properties of the biosilica scaffold: the morphology of the biosilica particles are known to vary significantly depending on the entrapped protein and upon changes of the reaction conditions.[29] These result are important as they demonstrate that a matrix-free approach is feasible,[28] yielding easier detection of the resonances of interest due to the higher filling factor. Notably, the buildup rates of the non-cryoprotected samples are higher than those of the cryoprotectant sample, suggesting that the biradical may have preferential interaction with the silica[20] and thus remain closer to the protein at the time of freezing (Table S1).

The DNP experiments at 5 T demonstrate the ability to polarize enzymes entrapped in biosilica. However, as expected for samples at cryogenic temperatures and low fields, the spectral resolution is low:[30,31] when protein samples are kept at cryogenic temperatures for the DNP measurements, broadening of the resonances is often observed. It is worth noting that in the existing literature on NMR studies at cryogenic temperature, lines are generally thought to broadened due to one of the following: a) freezing of different side-chain conformations that are no longer averaged by collisions with the solvent and/or intrinsic molecular motions;[30] b) interference of MAS and/or decoupling with molecular motions,[32] and c) differential interactions between the protein and the surrounding frozen solvent;[30] in particular for biosilica entrapped enzymes differential interactions with the biosilica network that are not averaged by molecular motions could also broaden the resonances.[10,11]

NMR at higher magnetic fields could mitigate the resolution problem. Thus, the immobilized enzymes were further analyzed at 16.4 T (699 MHz/460 GHz). Figure 1 shows the resulting DNP $^{13}C\{^1H\}$ CPMAS NMR spectra of the same samples: although the resolution is still not sufficient for a thorough characterization, this comparison shows that high field DNP provides a promising resolution improvement. Indeed, the present data demonstrate that even if the resolution is lower than the room temperature spectra, the DNP spectra clearly show resonances typical of the folded protein (around 160 ppm and 10 ppm, Figure S3), affording a method to assess the maintainment of the protein fold with higher sensitivity than the room-temperature SSNMR, thus with a lower detection limit.

From theory,[33] it is expected that the enhancement at 16.4 T will be at least 3.3 times smaller than the enhancement at 5 T. The AS-SOD sample follows such prediction (ε=15), while the enhancement of catMMP12 was reduced only by a factor ~2 (ε=16). This could be due to a phenomenon that has been described recently: the presence of dielectric discontinuities (*i.e.*, of particles with different dielectric constant) in the sample favors the dispersion of microwaves, which gives improved DNP performance.[34] Shorter wavelength microwaves could be scattered better, thereby accounting for the less-than-expected reduction of DNP at high field, with the different behavior between the two samples

ascribable to the different size of the biosilica particles (Figure S4) and possibly to the difference in microwave penetration. In biosilica-entrapped samples, the presence of microscopic particles is intrinsic, thus we can expect the higher DNP efficiency at high field to be a feature of such systems.

The enhancements at 16.4 T allowed for the collection of 2D experiments within a few hours using ~14–20 μl of sample. Figure 2 shows the improvement in resolution for the biosilica-entrapped AS-SOD enzyme when moving from 5 to 16.4 T (catMMP12 is shown in Figure S5). The comparison illustrates the importance of combining multidimensional experiments and high field DNP for improving both resolution and sensitivity; furthermore it is possible to foresee that using even higher fields would be beneficial for resolution, at the same time not compromising DNP efficiency too much. Figure S6 shows the comparison with the room temperature spectrum, reproduced with permission by the Royal Society of Chemistry from reference [12], which confirms that the peaks that appear at 16.4 T are present also at room temperature. On this basis, we can state that the modest resolution is due to low temperature effects, but the latter are mitigated at high field.

As already mentioned, it is possible to use positively charged proteins, such as hen egg-white lysozyme (HEWL) as catalysts.[4] DNP-enhanced NMR spectra of HEWL entrapped in the biosilica matrix in natural abundance were thus recorded (Figure 3). Whereas the spectrum without DNP shows no protein signals after ~5 hours, a difference spectrum with DNP clearly shows the presence of protein signals. This is striking considering the low concentration of non-labeled lysozyme in the sample. Previously, characterization of this system by SSNMR had only been possible via ^{29}Si.[35]

In conclusion high field DNP can be applied to biosilica-entrapped enzymes, and significantly higher sensitivity can be achieved, both with and without cryoprotectant. The ability to collect ^{13}C-^{13}C correlation spectra within hours of small sample volumes of AS-SOD and catMMP12 demonstrates the importance of DNP to achieve the sensitivity needed for studies of biosilica, bio-inspired materials, and of natural occurring materials (such as diatom's hexoskeleton) that are in natural abundance.[36,37] With continued study of sample preparation procedures at cryogenic temperatures, careful labeling procedures[10] and continued investment in radical design further improvements in resolution and sensitivity are possible. It is clear that DNP NMR will be required to mitigate or lift the sensitivity limitation that often restricts structural studies of complex biological systems.

Acknowledgments

A. Thakkar, J. Bryant, and M. Lucci are acknowledged for technical assistance. This work has been supported by Ente CR Firenze, the EC (Bio-NMR no. 261863), EU ESFRI Instruct, MIUR PRIN (2012SK7ASN); the NIH through the NIBIB (grants EB-003151, EB-002026, and EB-002804 and). E.R. acknowledges support from EMBO. V.K.M. is grateful to NSERC and the Government of Canada for a Banting Postdoctoral Fellowship.

References

1. Straathof AJJ, Panke S, Schmid A. Curr Opin Biotechnol. 2002; 13:548–556. [PubMed: 12482513]
2. Guisan, JM. Immobilization of Enzymes and Cells. Springer; 2006.

3. Poulsen N, Sumper M, Kröger N. Proc Natl Acad Sci. 2003; 100:12075–12080. [PubMed: 14507995]
4. Betancor L, Luckarift HR. Trends Biotechnol. 2008; 26:566–572. [PubMed: 18757108]
5. Müller WEG, Schröder HC, Burghard Z, Pisignano D, Wang X. Chem – Eur J. 2013; 19:5790–5804. [PubMed: 23512301]
6. Naik RR, Whitlock PW, Rodriguez F, Brott LL, Glawe DD, Clarson SJ, Stone MO. Chem Commun Camb Engl. 2003:238–239.
7. Otzen D. Scientifica. 2012; 2012:e867562.
8. Avnir D, Coradin T, Lev O, Livage J. J Mater Chem. 2006; 16:1013–1030.
9. Goobes G. Isr J Chem. 2014; 54:113–124.
10. Roehrich A, Drobny G. Acc Chem Res. 2013; 46:2136–2144. [PubMed: 23932180]
11. Wisser D, Brückner SI, Wisser FM, Althoff-Ospelt G, Getzschmann J, Kaskel S, Brunner E. Solid State Nucl Magn Reson. 2015; 66–67:36–39.
12. Fragai M, Luchinat C, Martelli T, Ravera E, Sagi I, Solomonov I, Udi Y. Chem Commun. 2013; 50:421–423.
13. Bertini I, Bhaumik A, De Paepe G, Griffin RG, Lelli M, Lewandowski JR, Luchinat C. J Am Chem Soc. 2010; 132:1032–1040. [PubMed: 20041641]
14. Knight MJ, Felli IC, Pierattelli R, Emsley L, Pintacuda G. Acc Chem Res. 2013; 46:2108–2116. [PubMed: 23506094]
15. Ni QZ, Daviso E, Can TV, Markhasin E, Jawla SK, Swager TM, Temkin RJ, Herzfeld J, Griffin RG. Acc Chem Res. 2013; 46:1933–1941. [PubMed: 23597038]
16. Lesage A, Lelli M, Gajan D, Caporini M, Vitzhum V, Mieville P, Alauzun J, Roussey A, Thieuleux C, Mehdi A, et al. J Am Chem Soc. 2010; 132:15459–15461. [PubMed: 20831165]
17. Michaelis VK, Ong TC, Kiesewetter MK, Frantz DK, Walish JJ, Ravera E, Luchinat C, Swager TM, Griffin RG. Isr J Chem. 2014; 54:207–221. [PubMed: 25977588]
18. Hall DA, Maus DC, Gerfen GJ, Inati SJ, Becerra LR, Dahlquist FW, Griffin RG. Science. 1997; 276:930–932. [PubMed: 9139651]
19. Song C, Hu KN, Joo CG, Swager TM, Griffin RG. J Am Chem Soc. 2006; 128:11385–11390. [PubMed: 16939261]
20. Lafon O, Thankamony ASL, Kobayashi T, Carnevale D, Vitzthum V, Slowing II, Kandel K, Vezin H, Amoureux JP, Bodenhausen G, et al. J Phys Chem C. 2013; 117:1375–1382.
21. Kiesewetter MK, Michaelis VK, Walish JJ, Griffin RG, Swager TM. J Phys Chem B. 2014; 118:1825–1830. [PubMed: 24506193]
22. Lelli M, Rossini AJ, Casano G, Ouari O, Tordo P, Lesage A, Emsley L. Chem Commun. 2014; 50:10198–10201.
23. Ravera E, Corzilius B, Michaelis VK, Rosa C, Griffin RG, Luchinat C, Bertini I. J Am Chem Soc. 2013; 135:1641–1644. [PubMed: 23331059]
24. Ravera E, Corzilius B, Michaelis VK, Luchinat C, Griffin RG, Bertini I. J Phys Chem B. 2014; 118:2957–2965. [PubMed: 24460530]
25. Ong TC, Mak-Jurkauskas ML, Walish JJ, Michaelis VK, Corzilius B, Smith AA, Clausen AM, Cheetham JC, Swager TM, Griffin RG. J Phys Chem B. 2013; 117:3040–3046. [PubMed: 23421391]
26. Mao J, Akhmetzyanov D, Ouari O, Denysenkov V, Corzilius B, Plackmeyer J, Tordo P, Prisner TF, Glaubitz C. J Am Chem Soc. 2013; 135:19275–19281. [PubMed: 24279469]
27. Corzilius B, Michaelis VK, Penzel SA, Ravera E, Smith AA, Luchinat C, Griffin RG. J Am Chem Soc. 201410.1021/ja5044374
28. Takahashi H, Lee D, Dubois L, Bardet M, Hediger S, De Paëpe G. Angew Chem. 2012; 124:11936–11939.
29. Patwardhan SV. Chem Commun Camb Engl. 2011; 47:7567–7582.
30. Siemer AB, Huang KY, McDermott AE. PLOS ONE. 2012; 7:e47242. [PubMed: 23077575]
31. Linden AH, Franks WT, Akbey Ü, Lange S, van Rossum BJ, Oschkinat H. J Biomol NMR. 2011; 51:283–292. [PubMed: 21826519]

32. ConcistrÈ M, Johannessen OG, Carignani E, Geppi M, Levitt MH. Acc Chem Res. 2013; 46:1914–1922. [PubMed: 23488538]
33. Hu KN, Debelouchina GT, Smith AA, Griffin RG. J Chem Phys. 2011; 134:125105. [PubMed: 21456705]
34. Kubicki DJ, Rossini AJ, Purea A, Zagdoun A, Ouari O, Tordo P, Engelke F, Lesage A, Emsley L. J Am Chem Soc. 2014; 136:15711–15718. [PubMed: 25285480]
35. Song X, Jiang Z, Li L, Wu H. Front Chem Sci Eng. 2014:1–9.
36. Gröger C, Lutz K, Brunner E. Prog Nucl Magn Reson Spectrosc. 2009; 54:54–68.
37. Bertermann R, Kröger N, Tacke R. Anal Bioanal Chem. 2003; 375:630–634. [PubMed: 12638046]

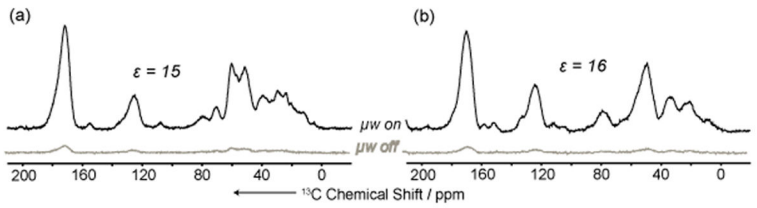

Figure 1.

700 MHz/460 GHz DNP enhanced $^{13}C\{^{1}H\}$ CPMAS NMR spectra with (top, 32 scans) and without (bottom, 256 scans) microwaves of biosilica encapsulated AS-SOD (a) and catMMP12 (b) in cryoprotectant and 10 mM TOTAPOL.

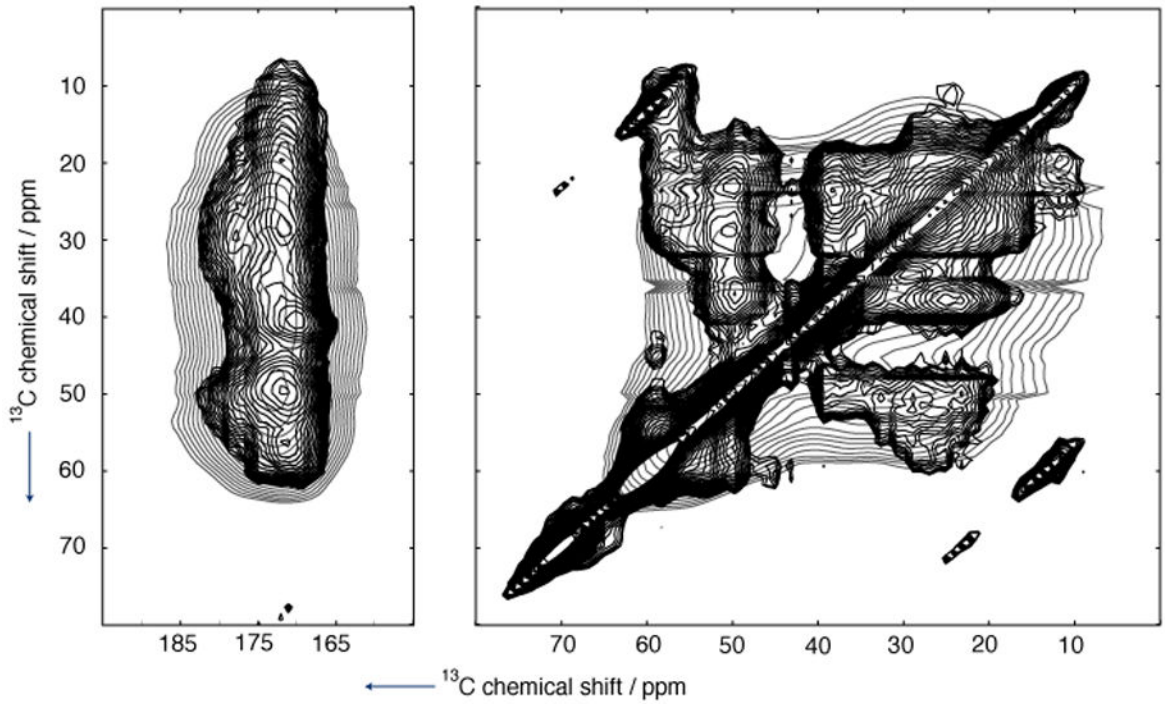

Figure 2.

Overlay of two-dimensional ^{13}C-^{13}C correlation spectra of AS-SOD at 5 T (gray, 45 μl sample volume, 20 ms mixing, 5 hrs. acquisition time) and 16.4 GHz (black, 14 μl sample volume, 50 ms mixing, 4 hrs. acquisition time) in cryoprotectant and 10 mM TOTAPOL.

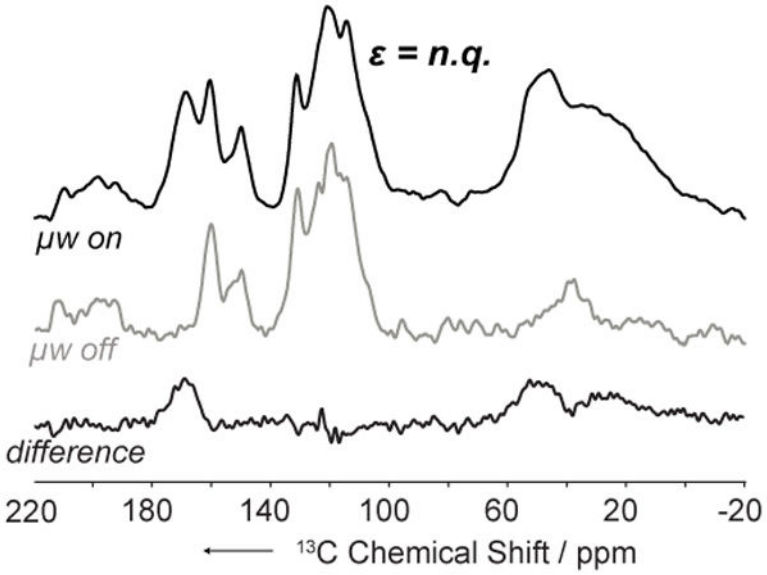

Figure 3.

$^{13}C\{^{1}H\}$ CPMAS DNP NMR of biosilica encapsulated lysozyme (natural abundance) with (top, black, 1,536 scans) and without (middle, gray, 4096 scans) microwaves recorded at 5 T. The bottom trace is a difference spectrum showing only protein resonances.