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Exploring Surfaces and Cavities in Lipoxygenase and Other Proteins by Hyperpolarized Xenon-129 NMR

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Abstract

This paper presents an exploratory study of the binding interactions of xenon with the surface of several different proteins in the solution and solid states using both conventional and hyperpolarized ^{129}Xe NMR. The generation of hyperpolarized ^{129}Xe by spin exchange optical pumping affords an enhancement by 3–4 orders of magnitude of its NMR signal. As a result, it is possible to observe Xe directly bound to the surface of micromolar quantities of lyophilized protein. The highly sensitive nature of the ^{129}Xe line shape and chemical shift are used as indicators for the conditions most likely to yield maximal dipolar contact between ^{129}Xe nuclei and nuclear spins situated on the protein. This is an intermediate step toward achieving the ultimate goal of NMR enhancement of the binding-site nuclei by polarization transfer from hyperpolarized ^{129}Xe. The hyperpolarized ^{129}Xe spectra resulting from exposure of four different proteins in the lyophilized, powdered form have been examined for evidence of binding. Each of the proteins, namely, metmyoglobin, methemoglobin, hen egg white lysozyme, and soybean lipoxygenase, yielded a distinctly different NMR line shape. With the exception of lysozyme, the proteins all possess a paramagnetic iron center which can be expected to rapidly relax the ^{129}Xe and produce a net shift in its resonance position if the noble gas atom occupies specific binding sites near the iron. At temperatures from 223 to 183 K, NMR signals were observed in the 0–40 ppm chemical shift range, relative to Xe in the gas phase. The signals broadened and shifted downfield as the temperature was reduced, indicating that Xe is exchanging between the gas phase and internal or external binding sites of the proteins. Additionally, conventional ^{129}Xe NMR studies of metmyoglobin and lipoxygenase in the solution state are presented. The temperature dependence of the chemical shift and line shape indicate exchange of Xe between adsorption sites on lipoxygenase and Xe in the solvent on the slow to intermediate exchange time scale. The NMR results are compared with N_2, Xe, and CH_4 gas adsorption isotherms. It is found that lipoxygenase is unique among the proteins studied in possessing a relatively high affinity for gas molecules, and in addition, demonstrating the most clearly resolved adsorbed ^{129}Xe NMR peak in the lyophilized state.

Introduction

Recent improvements in the technology for preparing hyperpolarized ^{129}Xe[1,2] have led to innovative applications in NMR, including studies of Xe bound to the surface of materials,[3,4] medical imaging of xenon in lungs[5] and blood,[6] and imaging of materials.[7,8] Fractional nuclear spin polarizations of greater than 70% have been achieved in small batch quantities of ^{129}Xe,[9,10] and with the advent of continuous flow optical pumping cells and high power diode laser arrays, large quantities of hyperpolarized gases can be produced and stored for

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hours.[11] The ^{129}Xe NMR signal enhancements are in the 10^3–10^5 range, and the immediate implication is that this enhancement might also be shared with other nuclei in a molecule or at a solid surface of interest. Indeed, a great deal of effort is currently being expended to optimize the conditions for efficient intermolecular polarization transfer. Success has been achieved using thermal mixing,[12] Hartmann-Hahn cross-polarization,[3] and intermolecular nuclear Overhauser effect in solution[7,13] and on solids,[4] but the potential for dramatic polarization transfer NMR enhancements of 4–5 orders of magnitude has yet to be realized. To date, maximum polarization transfer enhancements of up to 140–200 have been reported.[12] However, as the techniques for production of large volumes of highly polarized ^{129}Xe improve, and as better and more efficient polarization transfer conditions are realized, the diversity of applications will continue to expand.

In this paper we present the first results of the application of hyperpolarized ^{129}Xe NMR to lyophilized protein solids. By this method we explore the binding interactions of Xe to the surface of several different proteins in the solution and lyophilized states. The results are compared with N_2, Xe, and CH_4 gas adsorption isotherms. The experiments are intended to gain a better understanding of the Xe–protein interactions and to investigate the conditions under which maximal dipolar contact between the ^{129}Xe nuclei and nuclear spins situated in specific Xe binding sites on proteins can be achieved. Isoform 1 of soybean lipoxygenase (L-1), a protein with a large internal cavity,[14–16] and three other proteins in the lyophilized solid state, metmyoglobin (metMb), methemoglobin (metHb), and hen egg white lysozyme (Hel), are compared with respect to Xe binding. With the exception of Hel, these proteins all possess a paramagnetic iron center. In principle, a large paramagnetic shift of the ^{129}Xe NMR signal should be observed if the Xe is in exchange between the gas phase and a binding site in close proximity to the iron, and fast spin-lattice relaxation of the ^{129}Xe should also be evident. Of the proteins chosen for study, information about Xe binding is available from solution NMR and/or X-ray crystallography studies for metMb,[17–20] metHb,[17–20] and Hel,[21] but L-1 has not been studied previously with regard to Xe binding. In our solution state studies, we revisit the previously reported exchange of free Xe with Xe bound to metMb. In an L-1 solution, we observe an analogous exchange behavior, but with a transition from the slow to intermediate exchange regime occurring at higher temperature than in metMb solutions. The solution state data is compared with the ^{129}Xe NMR and gas adsorption isotherm data of the lyophilized state of these proteins.

Studies of Xe binding in proteins originated with the X-ray crystallographic work of Schoenborn et al.,[17] who demonstrated that Xe binds near the heme of metMb in the proximal cavity. Subsequent X-ray examination revealed other binding sites in metMb and in metHb.[18,20] Dozens of structures that include Xe bound at specific sites in cavities or pockets in proteins have been reported,[21] and these heavy atom sites are used for phasing in the X-ray structure determination. Access of Xe to cavities in proteins that functionally bind gas molecules is especially interesting. Solution state ^{129}Xe NMR studies of myoglobin and hemoglobin in various spin states provided further information on kinetic parameters of Xe binding.[19] Xenon line widths and chemical shifts were found to be functions of Xe gas pressure, protein concentration, spin state of the protein, and temperature. Analysis of the ^{129}Xe NMR signals in terms of multisite exchange provided association rates and binding activation energies. X-ray crystallographic studies at 270 K and 7 atm of Xe provided evidence of 4 Xe sites with fractional occupancies ranging from 0.45 to 1.0.[20] It was concluded[19,20] that the NMR experiments could distinguish two types of Xe sites on metMb and one on metHb. In some cases, it has been shown that Xe binding is not unique; other small molecules, such as cyclopropane[22] and benzene,[23] also bind to internal cavities in proteins.

Lipoxygenases catalyze the first step in oxidation of unsaturated fatty acids to form oxidation products involved in a variety of inflammatory responses.[24] Although structures of several

representatives of the L-1 family of enzymes exist,[14,15,24–28] there is only limited information about how substrates (unsaturated fatty acids and oxygen) interact with the large internal cavity. The iron ion involved in catalysis is located adjacent to the cavity. Thus far, one inhibitor[25] and several water molecules[23] have been located near or within the cavities of lipoxygenases by X-ray crystallography studies. The solid state ^{129}Xe NMR spectra and the gas adsorption isotherm data presented here show that lyophilized L-1 has a higher affinity than metMb, metHb, and Hel (per mole of protein) for Xe and several other gases.

Methods

Protein Samples.

Lipoxygenase (isoform L-1) was purified by a modification[29] of the procedure of Axelrod et al.[30] After an HPLC purification step, the protein was concentrated in a B-15 Minicon (Amicon, Beverly MA) to about 1.5 mM (142 mg/mL), dialyzed against 0.02 M TRIS (pH 7.5) for 12 hours, and lyophilized in a SpeedVac concentrator (Savant, Farmingdale, NY). MetHb (horse) and metMb (horse skeletal muscle) were purchased as lyophilized powders from Sigma (St. Louis, MO), and Hel (3× crystallized) was obtained as a lyophilized powder from Calbiochem (La Jolla, CA). The latter three proteins were dissolved and filtered (Millipore, 0.22 μm filter) to give solutions of 60 mg/mL in 0.02 M TRIS (pH 7.5) and lyophilized using procedures similar to those for L-1.

Generation of Hyperpolarized Xenon-129.

Hyperpolarized ^{129}Xe gas was prepared using the alkali atom noble gas spin-exchange optical pumping method.[1,31–33] Natural abundance Xe at a pressure of 0.20–0.60 atm was combined with a Rb metal droplet in a cylindrical Pyrex glass-pumping cell with a total volume of 80 cm^3. The temperature of the pumping cell was maintained at a temperature within 363–373 K in a magnetic field of approximately 0.010 T while the Rb vapor was excited with circularly polarized light at the wavelength corresponding to the ^{1}D line. The 1.75 Watt light beam was generated by an Argon-Ion (Coherent I-200) pumped Titanium Sapphire (Coherent 899) ring laser operated in multifrequency mode. This optical pumping apparatus typically produces a ^{129}Xe polarization of 2–4%, as determined from the enhancement factor over the ^{129}Xe signal obtained at thermal equilibrium from a reference sample containing a Xe/O_2 mixture of known Xe partial pressure.

NMR of Hyperpolarized Xenon-129 on Solid Samples.

The hyperpolarized ^{129}Xe spectra of Figure 3 have been acquired following exposure of 100–150 mg of each protein (metMb, metHb, Hel, and L-1) in the form of a lyophilized powder to 2.0 atm of ~2% spin polarized ^{129}Xe. The stated Xe pressure corresponds to the pressure measured at 298 K. The spectra of Figure 3 were acquired on a Varian Unity 500 MHz instrument. The additional hyperpolarized ^{129}Xe NMR spectra on L-1 presented in Figure 4 were acquired on a Bruker 400 MHz Advance NMR spectrometer at several temperatures between 173 and 223 K and at Xe pressures of 2.5 and 6.0 atm. On both spectrometers, the experiments were conducted using 10 mm high resolution liquid state NMR probes. In preparation for each hyper-polarized ^{129}Xe NMR run, Xe was loaded into the optical pumping cell and polarized for a period of > 10 min. The volume of the 10 mm medium wall pyrex NMR tubes used in the hyperpolarized NMR experiments is 8.5 ± 0.5 cm^3. To facilitate transfer of the hyperpolarized Xe from the pumping cell to the NMR tube containing the protein solid, and to also preserve the polarization of the hyperpolarized ^{129}Xe, the NMR tubes were immersed in a liquid nitrogen bath contained within the poles of an 0.2 T permanent magnet assembly. Following the freeze-over of the Xe, the tubes were sealed with a Teflon stopcock, removed from the vacuum manifold, and immediately transported to the NMR spectrometer. After inserting the sample into the NMR probe, the Xe was allowed to sublime for 120 s. The

acquisition was delayed for another 120 s to permit temperature equilibration. Each spectrum was acquired using a single 20-μs rf pulse corresponding to a 45° flip angle (the pulse width is sufficiently short to cover the full range of anticipated Xe chemical shifts). By using a flip angle of 45° rather than 90° the ^{129}Xe nuclear magnetization inside the NMR coil is only partially destroyed, allowing multiple spectra to be acquired. Exchange of xenon in and out of the coil region by diffusion also has the effect of replenishing the magnetization, so that the reduction of the ^{129}Xe NMR signal by the application of successive rf pulses is less than the factor $1\sqrt{2}$ that would result if there were no exchange. Since the coil volume of approximately 1 mL is much smaller than the total tube volume of 8.5 mL, several spectra (4–5) can be obtained without a significant loss in signal intensity. After obtaining a spectrum at the initial preset temperature, additional spectra were recorded at several lower temperatures. Following each temperature change, a 120-s delay was allotted to allow equilibration. To confirm that this delay was sufficiently long to achieve equilibration, a separate set of experiments was performed wherein spectra were acquired as a function of time following a temperature change. The lack of any time evolution after several minutes indicated that temperature equilibration was complete. Furthermore, the observed gas–liquid and liquid–solid phase transition temperatures were found to coincide well with values measured by conventional ^{129}Xe NMR in a reference sample containing a Xe–O_2 gas mixture which had been allowed to equilibrate for 30 min. The signals from liquid and solid ^{129}Xe occur at ~250 ppm (depending slightly on temperature) and ~300 ppm at 173 K with respect to the 298 K Xe gas signal at 0 ppm.

Xenon-129 NMR in Protein Solutions.

NMR spectra of ^{129}Xe dissolved in solutions of L-1 and metMb were recorded on the Bruker spectrometer fitted with a high-resolution, variable temperature 10-mm probe. The reported temperatures are accurate to within 2 K. The conventional (nonenhanced) spectra were recorded by signal averaging 6000 free induction decays (FIDs) with a recycle delay of 500 ms. Here again, the chemical shift scale was referenced to the 298 K Xe gas signal at 0 ppm.

The metMb sample consisted of 3.3 mM protein in a solvent composed of 50% D_2O, 25% methanol, and 25% ethylene glycol. This solution was degassed by three freeze-pump-thaw cycles. The spectra were recorded at a Xe pressure of 2.2 atm (at 298 K) over a temperature range of 248–308 K in 5 or 10 K increments. The concentration of the L-1 sample was 50 mg/mL in a solvent composed of 70% 0.01 M potassium phosphate buffer and 0.015 M sodium azide in 90% deuterium oxide and 30% glycerol. The L-1 solution was degassed gradually at ambient temperature until no further change in the pressure above the solution was detected. Spectra were recorded at a Xe pressure of ~1.7 atm in the 248–303 K range in 5 or 10 K increments.

Gas Adsorption Isotherm Measurements.

Methane and xenon gases were purchased from Matheson (Montgomeryville, PA), and N_2 was purchased from BITEC (Tampa, FL). All gases were 99.99% pure and were used as received. For the N_2 BET (Brunauer, Emmett, and Teller) and CH_4 gas adsorption isotherms, each protein powder solid was degassed for ~8 hours under a vacuum (10^{-4} Torr) at 77 K. Prior to recording the Xe adsorption isotherms, each protein powder solid was degassed for ~8 hours under a vacuum of 10^{-4} Torr at the following temperatures: L-1, 263 K; metMb, 293 K; and Hel, 293 K.

Nitrogen adsorption isotherms at 77 K were performed using a Micromeretics (Norcross, GA) ASAP 2000 gas analyzer. The BET surface areas were determined using a five-point BET calculation.[34,35] Gaseous uptake of CH_4 and Xe on the protein powder solids was measured at 210 K on the Micromeretics ASAP 2000 instrument with chemisorption/physisorption software employing a 75-point pressure table ranging from 0.1 Torr to 760 Torr. The system

was considered to be at equilibrium when the pressure change was less than 1% of the selected pressure point in a 20-s equilibration time interval. Pressure tolerances were 1% with the 10 Torr transducer and 1 mTorr with the 1000 Torr transducer. Low-temperature baths consisting of a solvent/ liquid nitrogen mixture were employed to collect isotherms at low temperatures.[36] The temperatures of the baths were maintained to within ±1 K.

A lyophilized L-1 sample that had been subjected to several rounds of Xe NMR experiments and to the adsorption isotherm measurements (over a period of several months) retained 50% of the original specific activity. Solution NMR experiments with L-1 were performed on a separate sample of specific activity 180 ± 10 μmol/min/mg. The activity of the protein confirmed that the integrity of the protein was preserved.

Results

Solution State Xenon-129 NMR.

The 110.7 MHz ^{129}Xe spectra in metMb solution, recorded as a function of temperature, are presented in Figure la. This series can be directly compared with the 27.7 MHz spectra of Tilton and Kuntz.[19] Note that we used myoglobin from a horse, while Tilton and Kuntz studied sperm whale myoglobin. The temperature dependence of the line width and the chemical shift is shown in Figure lb. A maximum line width of 860 Hz is observed at a temperature of 263 K. The data of Figure 1 exhibit a maximum upfield line shift of 7 ppm (3.3 mM metMb), while ~12 ppm was found in the original study at higher protein concentration (8 mM). Practically all features of the original ^{129}Xe NMR temperature dependence in metMb solution are well reproduced, albeit with higher dispersion and S/N due to improvements in NMR instrumentation since the original work. However, there was no discernible peak in the 0–300 ppm range that could be attributed to Xe bound to metMb in the slow to intermediate exchange limit.

The 110.7 MHz spectra of ^{129}Xe dissolved in a 50 mg/mL (0.5 mM) L-1 solution, recorded over a temperature range of 263–303 K, are presented in Figure 2a. A discontinuity in the chemical shift and line width appears at 263 K due to freezing of the 30% glycerol–water–protein solution. Because an unfolding transition of L-1 occurs at elevated temperatures[37] (T_m > 333 K), the upper limit of the temperature range investigated by solution NMR was restricted to 303 K. As shown in Figure 2b, both the line width and chemical shift vary nonlinearly with temperature and appear to reach maximum values near 303 K. These observations are consistent with Xe exchanging between the binding sites on the protein and the solvent in the slow to intermediate exchange regime. At no temperature within the range studied did we observe a second peak due to Xe bound to L-1 in the slow exchange regime.

Hyperpolarized Xenon-129 NMR Studies on Lyophilized Proteins.

Figure 3a–d presents the 138.4 MHz hyperpolarized ^{129}Xe NMR spectra of Xe in contact with lyophilized Hel, metMb, metHb, and L-1, respectively, at 193 and 223 K. The Xe pressure in each sample corresponded to 2.0 atm at 298 K. The 193 K spectrum of Figure 3c (middle) was obtained from a sample containing thermally polarized ^{129}Xe by signal averaging 2000 FIDs with a 1-s recycle delay. The resulting NMR line shape is consistent with the line shapes of the hyperpolarized ^{129}Xe spectra obtained at higher and lower temperature. Although the spectra derived from thermally polarized and hyperpolarization-enhanced ^{129}Xe have similar signal-to-noise ratios, it should be emphasized that the hyper-polarized ^{129}Xe spectra were acquired in only a single scan using a 45° rf pulse. This flip angle was chosen to yield useful spectra with good signal to noise while not fully destroying the nuclear magnetization of the hyperpolarized ^{129}Xe (as would be the case if a 90° pulse were applied). This made it possible to acquire the ^{129}Xe spectra at several different temperatures in a single optical pumping run.

The occurrence of exchange between surface-adsorbed and gas-phase xenon accompanied by diffusion has the effect of replenishing the xenon magnetization, especially at the higher temperatures, thus mitigating the destruction of the xenon magnetization by the successive rf pulses. Note that the vertical axes of the spectra in Figure 3 are normalized to the maximum intensity in each case. The spectra have not been scaled to account for the destruction of the finite, hyperpolarized xenon magnetization due to spin-lattice relaxation or due to the application of successive 45° rf pulses. The apparent variation in the signal-to-noise ratio for each temperature series depends not only on the history of rf pulses and delays applied to the sample, but also on amount of xenon adsorbed by the sample and the change in the NMR line width as the temperature is reduced. In view of the inability to obtain a good estimate of the effect of all these factors, we make no attempt here to interpret the relative intensities of the ^{129}Xe signals.

The line shapes presented in Figure 3 are qualitatively different for all four proteins. The 177 K spectrum of L-1 exhibits a particularly well-resolved protein-associated ^{129}Xe peak shifted downfield from the gas peak by about 35 ppm. In metHb, the broad component becomes resolved from the gas peak as the temperature is lowered, while in Hel and metMb the line shape broadens and becomes increasingly asymmetric. The spectra of metMb show an asymmetrically broadened peak with a tail extending downfield to about 40 ppm. The spectra at both temperatures were acquired following a single exposure of the protein to hyperpolarized Xe. After several minutes, the “bound” ^{129}Xe peak in the metMb sample decayed below the limit of detection, while the signal from the gas phase peak could be observed for as long as ~320 s. The NMR spectra of the metHb sample exhibited a resolved adsorption peak with a small (~2 ppm) downfield shift from the gas-phase peak. Note that the intensity of the gas-phase ^{129}Xe peak represents only a small fraction of the total amount of Xe in the lyophilized protein samples due to the displacement of the gas by the protein solid in the detected volume of the sample tube. The line width of the signal of ^{129}Xe bound to metHb was narrower (~5 ppm FWHM) than the line width of the signal obtained with metMb.

Adsorption of hyperpolarized ^{129}Xe onto lyophilized Hel yielded ^{129}Xe NMR peaks at ~5 ppm at 223 K and ~12 ppm at 183 K. The narrow peak due to gaseous Xe was not resolved in the Hel spectra. Unlike the other proteins studied, Hel is not paramagnetic, and hence the nuclear spin relaxation mechanism due to coupling with unpaired electrons is not present in Hel. This probably accounts for the comparatively higher intensity of the ^{129}Xe peak in Hel as well as its persistence for over 480 s.

The hyperpolarized ^{129}Xe NMR spectra of L-1 at several different temperatures and at Xe pressures corresponding to 2.5 and 6.0 atm at 298 K are presented in Figure 4. As in Figure 3, the spectra have been normalized to the maximum intensity, and no correction has been applied to account for the destruction of the finite, hyperpolarized xenon magnetization due to spin-lattice relaxation or due to the application of successive rf pulses. In the 2.5 atm series, a broad (~30 ppm FWHM), resolved peak develops at a temperature of 193 K. Upon lowering the temperature to 183 K, a strong peak appears at 250 ppm–evidence for a phase transition to liquid Xe. In the 6-atm series, the liquid Xe peak is initially observed at 223 K, with a shift of 230 ppm. The Xe gas–liquid phase transition temperatures at pressures corresponding to 2.5 and 6.0 atm at 298 K were independently established by conventional ^{129}Xe NMR on sealed Xe gas reference samples. The phase transition temperatures were, within error, the same in the gas reference and protein samples, indicating that the presence of protein does not substantially alter the thermodynamics of Xe liquification. The shifts in the positions of the adsorbed peaks and the changes in line width of the gas phase peak with temperature are consistent with a dynamic equilibrium between gas phase Xe and Xe adsorbed onto the protein surface.

Gas Adsorption Isotherms.

The binding of Xe, CH_4, and N_2 to the protein samples in the lyophilized state was also characterized by adsorption isotherm analysis. The adsorption of Xe on L-1 reaches a plateau near ~1.0 atm, suggesting near saturation of the adsorption sites, while the other proteins appear to continue to adsorb more Xe at pressures exceeding 1.0 atm (Figure 5). Table 1 summarizes the Xe adsorption data. On a per gram basis, all three proteins adsorbed similar amounts of Xe at 1.0 atm. However, on a per mole basis, L-1 adsorbs 25 Xe atoms per protein molecule at 1.0 atm pressure whereas metMb and Hel adsorb only about 4 moles of Xe per mole of protein. The plot in Figure 5 also demonstrates a lower affinity of L-1 and Mb for CH_4 in comparison with that of Xe. As reported in Table 1, the 77 K N_2 adsorption isotherms for L-1, Hel, and metMb yielded BET surface areas of 504, 23.3, and 13.0 m^2/g, respectively.

Discussion

The Xe adsorption isotherms at 210 K show that L-1, metMb, and Hel bind approximately the same number of moles of Xe per gram of protein at a Xe pressure of 1.0 atm (Figure 5). However, the curvatures of the binding isotherms demonstrate that L-1 approaches saturation at 1.0 atm, while in metMb and Hel, the Xe adsorption is still nearly linear with Xe pressure. This is consistent with a previous finding that the Xe adsorption by metMb saturates at much higher pressures. The Xe adsorption isotherm for a metMb/water solution at room temperature has been published,[38] and those experiments showed saturation as the Xe pressure approached 5.0 atm. The higher affinity of lyophilized L-1 for Xe per unit mole of protein is therefore evident, though not unique to this gas, since L-1 also has a higher affinity for methane relative to the other proteins examined (Figure 5). The N_2 BET surface areas, reported in Table 1, show that the available surface area for N_2 adsorption in L-1 is much higher (504 m^2/g) than in metMb, metHb, and Hel, per gram of protein. As a result of the smaller size of N_2 and the fact that the experiments were performed below the critical temperature of N_2, it may be concluded that N_2 has greater access to the internal surface structure and that N_2 liquification is involved in the adsorption process. The N_2 adsorption data differs from the Xe adsorption results in that, on a per gram basis, each protein adsorbs approximately the same amount of Xe (Table 1, Figure 5), though on a per mole basis, L-1 still has a much higher capacity for Xe. The dramatic difference in the N_2 and Xe adsorption behavior in L-1 indicates that no liquid Xe forms up to a pressure of 1.0 atm at 210 K, a finding supported by the lack of any liquid Xe NMR signal at this same temperature and at even higher pressures.

The following facts are relevant to the interpretation of the ^{129}Xe NMR spectra. The large chemical shift range of ^{129}Xe in different chemical environments affords a characterization of the Xe binding in both the solution and solid phases. Proteins can offer a large diversity of potential Xe binding sites, including surface exposed pockets, internal cavities or void spaces, and channel pores.[20] These sites may be lined with aliphatic, aromatic, or polar groups and could even involve reconfiguration or displacement of waters. Fluctuations in side-chain conformations are also thought to contribute to creating sites for Xe binding in proteins.[39] Although sites for Xe binding in proteins can be located by X-ray methods, NMR has the advantage of being able to characterize the binding dynamics in both the solid or solution phases. Polarization transfer from hyperpolarized ^{129}Xe to nuclei of residues near Xe binding sites in proteins will be particularly important in determining structure in proteins for which crystal structures are not available.

In the experiments on the lyophilized samples, each protein yielded a distinct ^{129}Xe NMR line shape (Figures 3 and 4). In the 183–223 K range, the NMR chemical shift of the adsorbed component varied from 0 to 40 ppm. The signals broadened and shifted downfield as the temperature was lowered. Although discrete peaks due to Xe bound to protein are not observed in metMb and Hel, the line shapes do become increasingly asymmetric as the temperature is

lowered, and the intensity of the downfield side of the line shape increases correspondingly. A reasonable explanation for this behavior is that the Xe is undergoing exchange between the gas and several unresolved sites associated with the surface or cavities. The broad tail of the line shape observed for metMb is consistent with the multiple (up to five)[20,39] sites detected in X-ray measurements of crystals and in calculations. The observation of a relatively narrow peak in metHb is consistent with a single type of Xe binding site, as reported previously[18] for this protein. X-ray studies of Hel crystals revealed two intramolecular Xe binding sites.[20] Although the Hel and metMb spectra (Figure 3) do not show fully resolved binding sites, the asymmetric shape of the peaks observed does suggest the presence of more than one type of binding site in these proteins.

The largest observed Xe chemical shift resulted from adsorption onto 142 mg of L-1 at a pressure corresponding to several atmospheres of Xe (at 298 K). Adsorbed peaks can be seen, at 10 ppm at 193 K and 35 ppm at ~177 K (Figure 3d and Figure 4b). The experimental conditions correspond to a large molar excess of Xe over protein. The internal cavity in soybean lipoxygenase-1 is referred to as cavity II, to distinguish it from a pocket known as cavity I that is open to the surface. Cavity II has two lobes and is lined by side chains of about 61 residues.[15,25] Calculations of the solvent-exposed surface area[40] of these side chains give an approximately linear increase from 10 to 1000 $Å^2$ for probe radii ranging from 2.5 to 1.0 Å, respectively. The accepted radius of Xe (2.1 Å) is on the large side of this range, but the calculations suggest that the exposed surface area in cavity II of L-1 could accommodate binding of multiple Xe atoms. The differences between L-1 and the other proteins, with respect to Xe binding, could also reflect the larger mass of L-1 (100 kD) which may provide a greater number of buried sites capable of accommodating a Xe atom than the smaller proteins of 14–17 kDa mass.

Results of the conventional (non-hyperpolarized) ^{129}Xe NMR study of metMb and L-1 solutions provide additional information. The previous observations of Tilton and Kuntz[19] demonstrated that Xe undergoes exchange between bound and free forms in the presence of metMb, with the bound signal exhibiting an upfield shift with respect to the chemical shift of Xe in solvent. They showed by extrapolation to infinite protein concentration that the chemical shift of ^{129}Xe bound to metmyoglobin in aqueous solution at 273 K is ~44 ppm from the signal of ^{129}Xe dissolved in solvent or about 153 ppm downfield from the gas-phase ^{129}Xe signal. The ^{129}Xe signal in the metMb solution is broadest at about 264 K and is sharper above and below this temperature. In agreement with this prior study, our data is consistent with a transition from the fast to intermediate exchange regime near 264 K, but at 248 K, we were not able to detect the second broad signal attributed by Tilton and Kuntz to the bound component in slow exchange. It should be noted that our measurements are made at about four times higher Larmor frequency and at one-third lower protein concentration than in the original study. Under our conditions, increased paramagnetic broadening at higher magnetic field may render the bound signal more difficult to observe than in the lower field study of the original work, and in addition, the lower protein concentration in our work would further diminish the possibility of detecting the bound signal. The temperature at which the transition from rapid to slow exchange occurs is approximately the same in both experiments, a finding consistent with a small activation energy for the exchange. Chemical shifts of Xe in the solvent alone (50% D_2O, 25% methanol, 25% ethylene glycol) do not reveal any phase transitions between 273 and 303 K.[19]

An alternative interpretation of the broadening of peaks in the metMb solution over the 278–253 K range (Figure la) is that metMb undergoes “cold denaturation” in this range.[41] Under that interpretation, two forms of protein, native and unfolded, are in equilibrium, and Xe is in rapid exchange between the solvent and the two protein environments. Studies of cold denaturation of metMb[41] show that a transition at about 263 K would be associated with a pH

around 4 rather than the pH of our experiment (pH25°C 7.4, 0.02 M TRIS). However, cold denaturation of metMb has not been examined in the cryoprotectant solvent system used in our experiments, so further studies are necessary to fully rule out this alternative explanation for the observed temperature dependence of the line width and line shift in the solution-state ^{129}Xe NMR.

In lipoxygenase solutions, increasing the temperature from 268 to 303 K results in an increase in the ^{129}Xe NMR line width and a downfield change of the chemical shift. Higher temperatures were not studied to avoid thermal denaturation of L-1.[37] The sign of the change in chemical shift is opposite to that observed in metMb, whereas the signal in the fast exchange limit is shifted upfield with respect to ^{129}Xe in solvent in metMb. In metHb, on the other hand, Tilton and Kuntz report a downfield shift in the solution state. These differences in the direction and magnitude of the paramagnetic shift change may be attributed to differences in the relative orientation and proximity of Xe to the unpaired electron in these paramagnetic proteins. No peak that might be attributed to Xe bound to L-1 at low temperature was observed over the 0–300 ppm range. Again, this could be due to several factors, such as a low fractional occupancy of the bound state at 1.7 atm, the relatively low protein concentration, or severe paramagnetic broadening of the bound ^{129}Xe signal.

Conclusions

In summary, we have presented the first hyperpolarized ^{129}Xe NMR spectra obtained in the presence of lyophilized protein powders–soybean lipoxygenase, methemoglobin, metmyoglobin, and hen egg white lysozyme. The ^{129}Xe NMR signal enhancement afforded by spin exchange optical pumping dramatically illustrates the advantage of using hyperpolarized ^{129}Xe in protein studies. While it is possible to obtain adequate ^{129}Xe spectra with considerable signal averaging in the case of a paramagnetic protein such as methemoglobin, this will probably not be practical for diamagnetic proteins such as lysozyme. Moreover, we have demonstrated that it is possible to obtain reproducible spectra at several different temperatures by using the large reservoir of nuclear polarization generated in a single optical pumping run.

In the lyophilized state, each protein exhibited a distinct and reproducible ^{129}Xe NMR line shape. The small chemical shifts observed suggest the peaks are due to surface-bound Xe in exchange with gaseous Xe. The protein-associated peak in lipoxygenase was more distinct than in the other proteins. This could be attributed to the large channel pore in this particular protein–an explanation that is consistent with the greater BET surface area and greater Xe uptake as observed by independent gas adsorption measurements. Polarization transfer experiments could help to unambiguously resolve this issue.

The Xe phase diagram ultimately constrains the pressure and temperature range over which the protein-bound ^{129}Xe signals can be observed. If the pressure is too high or the temperature too low, Xe condenses into a bulk phase that yields no information about the Xe–protein interaction and will not produce effective polarization transfer to nuclei of the protein. Our hyperpolarized ^{129}Xe studies reveal two possible strategies for observing Xe bound to internal cavities in lyophilized proteins, and these conditions are likely to match the conditions needed for optimal dipolar contact with the nuclei situated at the Xe binding sites. In the first approach, a relatively high pressure and high temperature achieve a high fractional occupancy of the Xe binding. Alternatively, the adsorption may be done at relatively low pressure and low temperature, where the hyperpolarized ^{129}Xe will not condense into a bulk phase and will be present at a concentration comparable to the density of Xe binding sites on the protein. The first approach should even be amenable to studies of paramagnetic proteins because the Xe will be in fast exchange with excess polarized Xe in the gaseous state. In the latter approach,

it is clear that a higher Xe polarization will be required, and the immobilized Xe will experience rapid spin-lattice relaxation if the binding site is in close proximity to a paramagnetic center.

Although our experiments were conducted with ^{129}Xe polarized to only 2–4%, the results suggest that it may be possible to achieve polarization transfer in lyophilized proteins with higher levels of polarization and/or continuous flow of hyperpolarized ^{129}Xe across a solid sample. Lysozyme is an ideal candidate for such an experiment because it is diamagnetic and exhibits both X-ray and NMR evidence for Xe binding. Lysozyme can accommodate an isotopically enriched substrate molecule that could also be the target of polarization transfer enhancement. Competitive binding studies can also be envisaged. For instance, if Xe is found to be competitive with O_2 binding in L-1, then it may be possible to trap an enzymatic intermediate state involving simultaneous binding of both Xe and the fatty acid substrate. The existence of such an intermediate could be proven by demonstrating polarization transfer from the hyperpolarized ^{129}Xe to the ^{1}H or ^{13}C nuclei of the substrate.

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References

1. Cates GD, Fitzgerald RJ, Barton AS, Borgorad P, Gatzke M, Newbury NR, Saam B. Phys. Rev. A At., Mol., Opt. Phys 1992;45:4631–4639.
2. Raftery D, Long H, Meersman T, Grandinetti PJ, Reven L, Pines A. Phys. Rev. Lett 1991;66:584–587. [PubMed: 10043847]
3. Long HW, Gaede HC, Shore J, Reven L, Bowers CR, Kritzebberger J, Pietrass T, Pines A. J. Am. Chem. Soc 1993;115:8491–8492.
4. Brunner E, Seydoux R, Haake M, Pines A, Reimer JA. J. Magn. Res 1998;130:145–148.
5. Albert MS, Cates GD, Driehuys B, Happer W, Saam B, Springer CS Jr, Wishnia A. Nature (London) 1994;370:199–201. [PubMed: 8028666]
6. Wolber J, Cherubini A, Dzik-Jurasz ASK, Leach MO, Bifone A. Proc. Natl. Acad. Sci. U.S.A 1999;96:3664–3669. [PubMed: 10097094]
7. Navon G, Song YQ, Room T, Appelt S, Taylor RE, Pines A. Science (Washington, D.C.) 1996;271:1848–1851.
8. Tseng CH, Wong GP, Pomeroy VR, Mair RW, Hinton DP, Hoffmann D, Stoner RE, Hersman FW, Cory DG, Walsworth RL. Phys. Rev. Lett 1998;81:3785–3788. [PubMed: 11543589]
9. Ruth U, Hof T, Schmidt J, Fick D, Jänsch HJ. Appl. Phys. B 1999;68:93–97.
10. Jänsch HJ, Hof T, Ruth U, Schmidt J, Stahl D, Fick D. Chem. Phys. Lett 1998;296:146–150.
11. Gatzke M, Cates GD, Driehuys B, Fox D, Happer W, Saam B. Phys. Rev. Lett 1993;70:690. [PubMed: 10054178]
12. Bowers CR, Long HW, Pietrass T, Gaede HC, Pines A. Chem. Phys. Lett 1993;205:168–170.
13. Fitzgerald RJ, Sauer KL, Happer W. Chem. Phys. Lett 1998;284:87–92.
14. Gaffney BJ. Annu. Rev. Biophys. Biomol. Struct 1996;25:431–459. [PubMed: 8800477]
15. Boyington JC, Gaffney BJ, Amzel LM. Science (Washington) 1993;260:1482–1486. [PubMed: 8502991]
16. Minor W, Steczko J, Bolin JT, Otwinowski Z, Axelrod B. Biochemistry 1993;32:6320–6323. [PubMed: 8518276]
17. Schoenborn BP, Watson HC, Kendrew JC. Nature (London) 1965;207:28–30. [PubMed: 5893727]
18. Schoenborn BP. J. Mol. Biol 1969;45:297–303. [PubMed: 5367030]
19. Tilton RF Jr, Kuntz ID Jr. Biochemistry 1982;21:6850–6857. [PubMed: 7159568]

20. Tilton RF Jr, Kuntz ID Jr, Petsko GA. Biochemistry 1984;23:2849–2857. [PubMed: 6466620]
21. Prange T, Schiltz M, Pernot L, Colloc'h N, Longhi S, Bourguet W, Fourme R. Proteins: Struct., Funct., Genet 1998;30:61–73. [PubMed: 9443341]
22. Schoenborn BP. Nature (London) 1967;214:1120–1122. [PubMed: 6059504]
23. Feher VA, Baldwin EP, Dahlquist FW. Nat. Struct. Biol 1996;3:516–521. [PubMed: 8646537]
24. Samuelsson, B., Ramwell, P. W., Paoletti, R., Folco, G., Granström, E., Nicosia, S., Eds: *Advances in Prostaglandin, Thromboxane and Leukotriene Research*, v. 23; Raven Press: New York, 1995.
25. Minor W, Steczko J, Stec B, Otwinowski Z, Bolin JT, Walter R, Axelrod B. Biochemistry 1996;35:10687–10701. [PubMed: 8718858]
26. Skrzypczak-Jankun E, Amzel LM, Kroa BA, Funk MO. Proteins: Struct. Funct. Genet 1997;29:15–31. [PubMed: 9294864]
27. Gillmor SA, Villasenor A, Fletterick R, Sigal E, Browner MF. Nat. Struct. Biol 1997;4:1003–1009. [PubMed: 9406550]
28. Prigge ST, Boyington JC, Gaffney BJ, Amzel LM. Proteins: Struct., Funct., Genet 1996;24:275–291. [PubMed: 8778775]
29. Gaffney BJ, Mavrophilipos DV, Doctor KS. Biophys. J 1993;64:773–783. [PubMed: 8386016]
30. Axelrod B, Cheesbrough TM, Laakso S. Methods Enzymol 1981;71:441–451.
31. Bhaskar ND, Happer W, McClelland T. Phys. Rev. Lett 1982;49:25.
32. Happer W, Miron E, Schaefer S, van Wijngaarden WA, Zeng X. Phys. Rev. A 1984;29:3092–3110.
33. Zeng X, Wu Z, Call T, Miron E, Schreiber D, Happer W. Phys. Rev. A 1985;31:260–278. [PubMed: 9895475]
34. Brunauer S, Emmett PH, Teller E. J. Am. Chem. Soc 1938;60:309.
35. Drago RS, Webster CE, McGilvray JM. J. Am. Chem. Soc 1998;120:538–547.
36. Gordon, A. J.; Ford, R. A. *The Chemist's Companion;* John Wiley and Sons: New York, 1972, p 451.
37. Gaffney BJ, Sturtevant JM, Yuan SM, Lang DM, Dagdigian E. Biophys. J 1994;66:A178.
38. Ewing GJ, Maestas S. J. Phys. Chem 1970;74:2341–2344. [PubMed: 5445459]
39. Tilton RF Jr, Singh UC, Weiner SJ, Connolly ML, Kuntz ID Jr, Kollman PA. J. Mol. Biol 1986;192:443–456. [PubMed: 3560222]
40. Fraczkiewicz R, Braun W. J. Comput. Chem 1998;19:319–333.
41. Privalov PL. Annu. Rev. Biophys. Biophys. Chem 1989;18:47–69. [PubMed: 2660833]

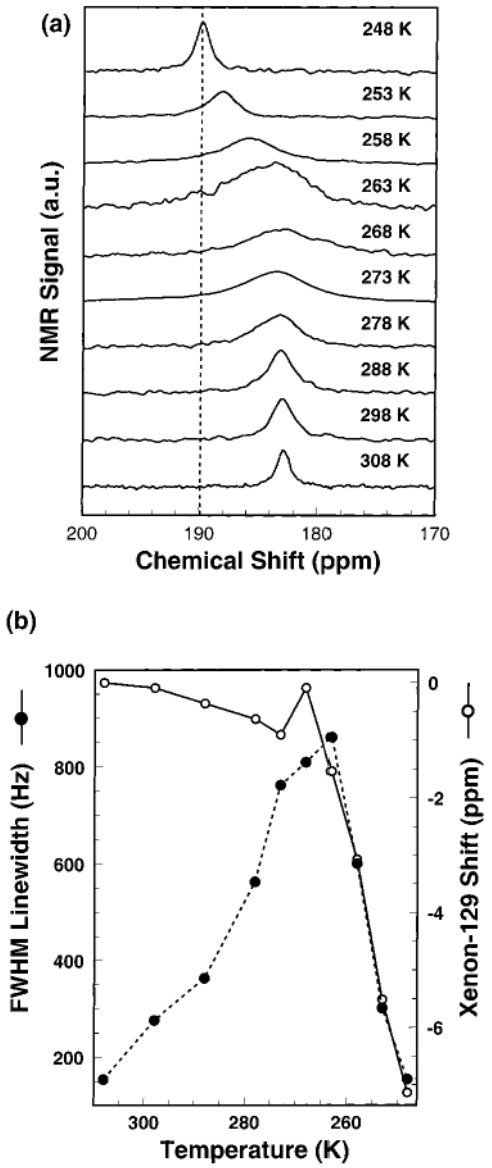

Figure 1.

(a) Conventional ^{129}Xe NMR spectra, acquired as a function of temperature, of a solution containing 3.3 mM metMb in 0.50 mL of ethylene glycol, 0.50 mL of methanol, and 1.0 mL of D_2O. The 8.5-mL volume sample tube contained 2.2 atm of xenon. Fourier transformed spectra were acquired by signal averaging 6000 FIDs with a 500-ms recycle delay. (b) Summary of the temperature dependence of the ^{129}Xe NMR line widths (filled circles) and line shifts (open circles) in the metMb sample.

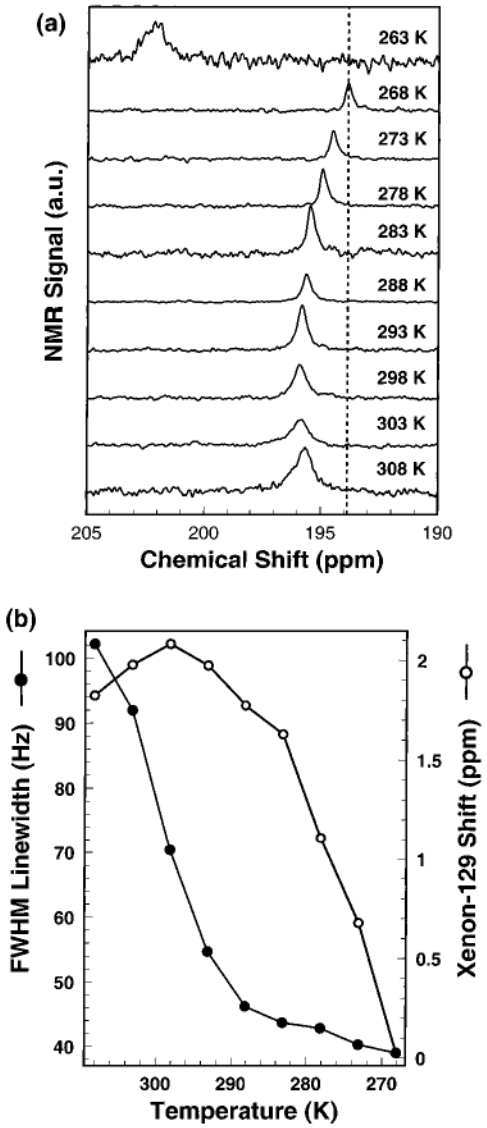

Figure 2.

(a) Conventional ^{129}Xe NMR spectra of a 1 mL solution containing 50 mg of lipoxygenase, type L-1 from soybeans, dissolved in a 0.01 mM potassium cryoprotectant solvent (see text). The spectra were acquired as a function of temperature at a Xe pressure of approximately 1.7 atm. A total of 4000 FIDs were signal-averaged using a recycle delay of 500 ms. (b) Summary of the temperature dependence of the ^{129}Xe NMR line widths (filled circles) and line shifts (open circles) in the L-1 sample.

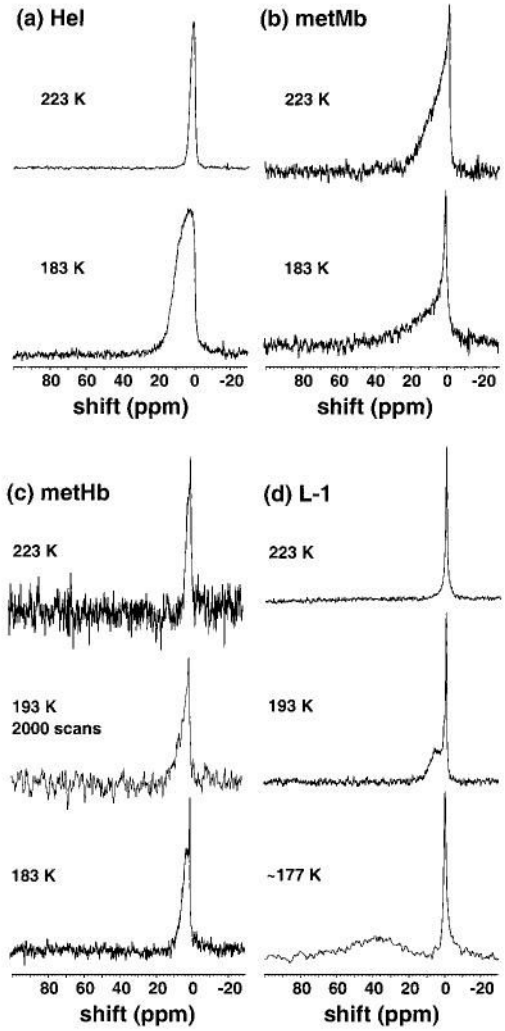

Figure 3.

Hyperpolarized ^{129}Xe NMR study of Xe adsorption on 100–150 mg samples of lyophilized powders of (a) hen egg white lysozyme (Hel), (b) metmyoglobin (metMb), (c) methemoglobin (metHb), and (d) soybean lipoxygenase (L-1) at several temperatures, as indicated. The spectra have been normalized to the maximum intensity in each spectrum, and no correction has been applied to account for the loss of xenon spin magnetization by spin lattice relaxation or rf pulses. Except for the the 193 K spectrum of part (c), the ^{129}Xe NMR spectra were all recorded at 138.4 MHz using a single 45° pulse (20-μs pulse width) in the presence of hyperpolarized xenon gas at a pressure equivalent to 2.0 atm at room temperature. The samples were thermally equilibrated for several minutes prior to acquisition. The 193 K spectrum of (c) is a conventional (field-polarized) ^{129}Xe NMR spectrum acquired at 110.7 MHz by signal averaging 2000 FIDs with a 1-s recycle delay.

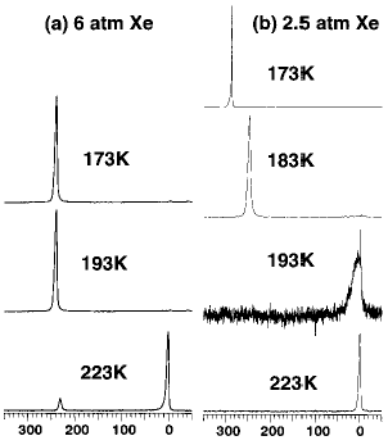

Figure 4.

Hyperpolarized ^{129}Xe NMR study of Xe adsorption on 142 mg of lyophilized soybean lipoxygenase (L-1) exposed to (a) 2.5 atm (at room temperature) and (b) 6.0 atm of hyperpolarized Xenon. At each pressure, spectra are shown for several different temperatures. The sample was thermally equilibrated for several minutes prior to recording the 110.7 MHz spectra in a single scan following a 45° pulse. The spectra have been normalized to the maximum intensity in each spectrum, and no correction has been applied to account for the loss of xenon spin magnetization by spin-lattice relaxation or rf pulses.

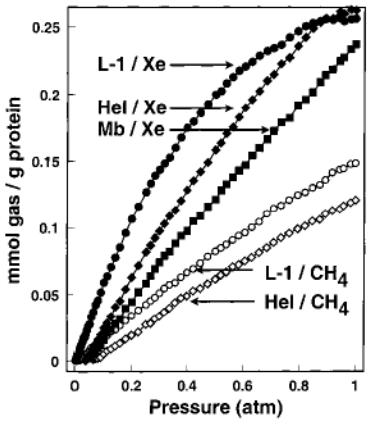

Figure 5.
Xe and CH_4 adsorption isotherms for binding at 210 K to lyophilized proteins, lipoxygenase (L-1), metmyoglobin (metMb), and hen egg white lysozyme (Hel). Adsorption data is also reported in Table 1.

Table 1

Parameters from Adsorption Isotherm Data for Lyphilized Proteins

protein	N_2 BET surface area (m^2/g)	mmol Xe adsorbed per g of protein[a]	mol Xe adsorbed per mol protein[a]
Hel	13.0 ± 0.7	0.262	3.67
metMb	23.3 ± 3.6	0.237	4.00
L-l	504 ± 9	0.256	25.1

[a] At a pressure of 1 atm.