Experimental Measurement of Nonuniform Dioxygen Accessibility to Ribonuclease A Surface and Interior

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Molecular accessibility to protein surfaces and interiors is fundamental to molecular recognition and biological function. Structures derived from diffraction experiments suggest that molecular access to the functional sites in some proteins, myoglobin for example, requires significant protein rearrangement. Nevertheless, the fact that oxygen binds rapidly to myoglobin supports molecular dynamics simulations which suggest that sufficient structural fluctuations are present to provide little barrier to oxygen binding to the heme.[1,2] A remarkable feature of these simulations is the possibility that oxygen may penetrate the protein structure through pathways that appear to be sterically prohibited. This work is motivated by the desire to obtain high resolution measures of molecular accessibility to different structural regions of a folded protein. We use molecular oxygen as the accessibility probe.

Measures of molecular accessibility include chemical reactivity, amide−hydrogen exchange kinetics, fluorescence quenching, and nuclear magnetic resonance contact shifts and relaxation. The fluorescence quenching experiment,[3] while it provides both spatial and dynamic information, suffers from the small number of reporter sites. The nuclear magnetic relaxation experiment, which suffers from dramatically decreased detection sensitivity, may utilize one or more reporter spins on each amino acid. A fundamental difference between the fluorescence and NMR experiments is the time scale for the observation. The fluorescence quenching experiments sense events on the time scale of the fluorescence lifetimes, which are often in the range of tens of nanoseconds. For the NMR experiments reported here, the oxygen-induced effects accumulate over the spin−lattice relaxation time of the observed spin, which is somewhat longer than 0.1 s.

The paramagnetic contribution to the observed proton spin−lattice relaxation rates is proportional to the square of the electron−nuclear magnetic dipole−dipole coupling strength between the paramagnetic oxygen center and the observed nuclear spin. Oxygen is unique in that the electron spin−lattice relaxation time is of order 10 ps and is nearly independent of solvent or local environment provided that the oxygen does not chemically react as it does in hemoglobin.[4] Because the oxygen spin−lattice relaxation time is shorter than most inter- and intramolecular correlation times such as rotation and translation, the effective correlation time for the electron−nuclear coupling is constrained by the oxygen T_{1e}. This fact simplifies the analysis of oxygen-induced relaxation rates in a protein considerably because the correlation time for the inter-moment coupling is essentially the same whether the oxygen diffuses at the protein surface or penetrates into the folded structure of the protein.

The difficulty the short T_{1e} creates is that the paramagnetic contributions to the relaxation rate may be small because the correlation time is small and in the extreme narrowing limit the relaxation rate contribution is linear in the correlation time. However, the paramagnetic contributions to nuclear spin relaxation may be adjusted because they depend linearly on the electron-spin concentration; 10 atm of oxygen provides a significant change in the relaxation rates of most protein protons in the present experiments.

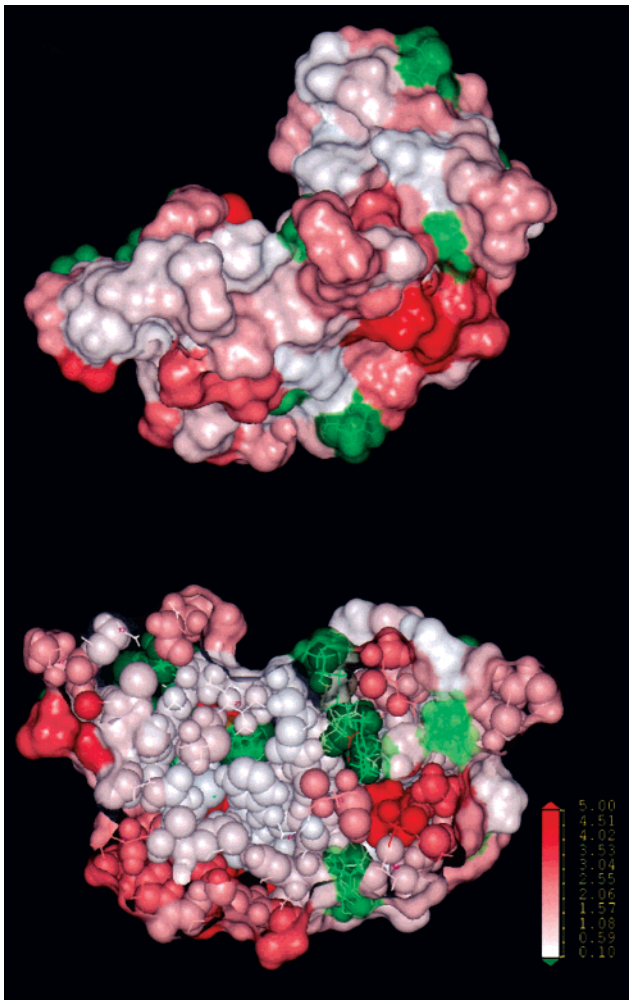

Figure 1. A space filling model structure for ribonuclease A that is colored based on the oxygen-induced changes in the proton nuclear spin−lattice relaxation rates. The relaxation rate differences derive from two experiments: one data set taken at 10 atm of oxygen and one data set taken on an oxygen-free sample recorded under 1 atm of nitrogen. The greatest changes are indicated in red, the smallest changes in white. A green region indicates that no resonance was cleanly resolved to provide an unambiguous relaxation rate difference with the modified double quantum filtered COSY experiment employed. Each data set was recorded using a sweep width of 5 kHz digitized in 1024 complex points and apodized with a 90° shifted sinebell function and zero-filled to 2048 complex points. Sixteen acquisitions were added for each of 256 t1 increments which were apodized with a similar sinebell function and zero filled to 1024 complex points. The red-scale covers the range of relaxation rate changes from 0.10 s^{-1}, which is white, to 5.0 s^{-1}, which is deepest red. The top view represents the outside surface of the molecule, and the bottom view is a cross-section through a different orientation of the molecule to show the inside distribution of oxygen accessibility.

The strategy is to measure the oxygen-induced contribution to proton spin−lattice relaxation rates throughout the structure of the protein. If no binding interactions are important and relative intermolecular diffusion is rapid, then the dipole−dipole relaxation rate is related to the distance of closest approach between the oxygen−electron magnetic moment and the observed proton.[5] If the oxygen binds to the protein and is temporarily trapped at a

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particular binding site, the correlation time for the coupling remains the oxygen T_{1e}, but the relaxation rate is proportional to the inverse sixth power of the distance.[6] In either case, the change in relaxation rate reports the close proximity of molecular oxygen to the observed proton.

Figure 1 shows a representation of the ribonuclease A structure.[7] The color scale summarizes the differences in proton spin−lattice relaxation rates obtained under 10 atm of oxygen and under 1 atm of nitrogen. A double quantum filtered COSY sequence provided the structural resolution using a Varian Unity Plus NMR spectrometer operating at 500 MHz. Spectral assignments were taken from earlier work.[8,9] Although there are more time-efficient data acquisition approaches,[10] the relaxation-rate differences are proportional to the strength of the proton−oxygen magnetic dipole−dipole coupling. A residue that provided no well-resolved cross-peaks is colored green. The remaining residues are placed on a color scale that ranges from white to dark red covering a relaxation rate change of 0.1 to 5 s^{-1}. If there is a relaxation rate change of 5 s^{-1} that portion is colored red; if there is a relaxation rate change of 2.5 s^{-1}, that portion is colored pink, etc. The reddest regions experience the greatest effective interaction with oxygen, the white regions the least. The top structure shows the ribonuclease A surface, and the bottom structure is a cross-section through a different orientation.

We make several observations based on these data; a more detailed analysis of this experiment will be presented subsequently. If oxygen sampled the protons observed by random hard-sphere collisions determined by the relative diffusive motions, the relaxation rate change computed from the Freed formalism would be 2.5 s^{-1} assuming a distance of closest approach of 2.5 Å and a relative translational diffusion constant of 0.6×10^{-5} cm^2 s^{-1}.[11] This calculation assumes a point dipole approximation for two spins; however, the COSY experiment that provides the resolution in this case involves two protons simultaneously in the relaxation monitored, which decreases the spatial resolution somewhat. Nevertheless, the dynamic range of the experiment is good. The average relaxation rate observed in the diamagnetic solution is 0.43 s^{-1}; the average rate for the paramagnetic case is 2.68 s^{-1}. Figure 1 shows that the access of oxygen to the observed protons is not uniform, even on the surface. Proton−proton cross-relaxation may couple the relaxation of protons in close proximity and cause spins to have nearly identical rates, particularly if they relax slowly. However, the effects of cross-relaxation do not dominate these observations because Figure 1 shows that even adjacent residues may have very different effective coupling to the oxygen. The oxygen effects are somewhat larger for the surface residues than the interior residues. However, relaxation of protons in the protein interior is readily observed, which is consistent with the effectiveness of oxygen in quenching fluorescence of buried tryptophan residues and NMR experiments that demonstrate low molecular weight hydrocarbons may fill protein cavities.[12] The uneven oxygen accessibility to the protein interior suggests that the fluctuations that permit oxygen contact involve motions of loosely coupled structural domains in the folded protein. The uneven oxygen accessibility at the surface appears to reflect different distances of closest approach that may be modulated by water occupancy at solvation sites and counterions sterically inhibiting close approach. A high water molecule or counterion occupancy will increase the oxygen−protein contact distance, on average, and decrease the oxygen contribution to the protein−proton relaxation rate. Thus, surface heterogeneity indicated in Figure 1 may reflect in part the effective solvation structure of the protein surface as experienced by a freely diffusing neutral oxygen molecule.

This experimental approach may be easily extended to other nuclei such as ^{15}N to gain data acquisition efficiency and spectral and spatial resolution. It provides a useful experimental means for exploring intermolecular contacts between a small neutral molecule and proteins in solution.

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Supporting Information Available: Table of experimental data and figures showing the gradient double-quantum COSY pulse sequence and representative relaxation curves (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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