High-pressure conformational ensemble of apomyoglobin revealed by double electron-electron resonance

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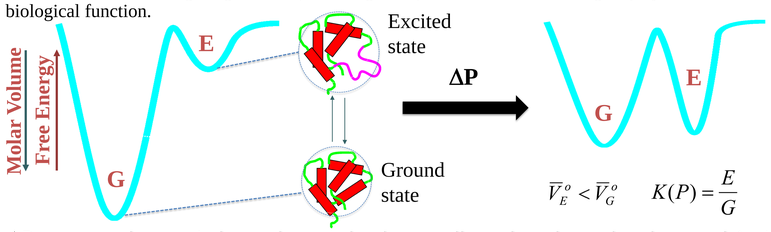
Abstract

The dominance of a well-ordered native state at ambient conditions for most proteins belies the functional importance of conformational fluctuations on a wide range of time and length scales. Higher energy conformational states (excited states) may play an important functional role, yet are too sparsely populated to allow spectroscopic investigation. Perturbation techniques such as high hydrostatic pressure may be employed to increase the population of excited states for study, but structural characterization is not trivial, due to the multiplicity of states in the ensemble and rapid (μs-ms) conformational exchange. The method of Site-Directed Spin Labeling (SDSL) in combination with Double Electron-Electron Resonance (DEER) is ideally suited for this purpose. DEER spectroscopy on spin-labeled protein provides long range (2-8 nm) and discrete distance distributions in heterogeneous systems with angstrom-level resolution, but must be carried out at cryogenic temperatures. In order to study the high pressure conformational ensemble of proteins, we developed a method for rapidly freezing spin-labeled proteins under pressure. This kinetically traps the high pressure equilibrium for subsequent data acquisition by DEER at atmospheric pressure and cryogenic temperature. We evaluated this methodology using seven doubly-labeled mutants of myoglobin designed to monitor discrete inter-helical distances in the protein. For holomyoglobin, the distance distributions are narrow and relatively insensitive to pressure, reflecting the insensitivity of the spin-label internal motion to pressure and the absence of low-lying excited states in the energy landscape of the holo protein. On the other hand, a distinct pattern of pressure-dependent changes in apomyoglobin in the range of 0 – 3000 bar signal the appearance of a molten globule state involving increased conformational fluctuations of specific helices within an otherwise folded structure. A direct comparison of the pressure- and pH-induced molten globules reveals key differences in the amplitude of motion sampled by each helix.

Thermodynamics and structure of proteins under pressure

❖Although a well-ordered native state is dominant for most proteins under physiological conditions, excited states involving large-scale (multi-angstrom) conformational changes play critical roles in biological function.



❖Pressure populates excited states because they have smaller molar volumes than the ground (native) state[1], as illustrated above. The pressure-induced reduction in molar volume is achieved through elimination of packing defects, electrostriction of charged residues, and hydration of buried residues[2].

❖The excited state ensemble may be heterogeneous, consisting of a manifold of substates, making determination of pressure-induced structural changes difficult.

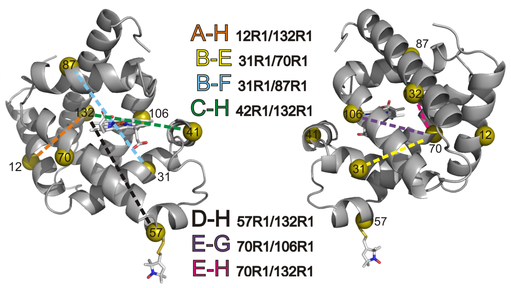
❖Double Electron-Electron Resonance (DEER) spectroscopy coupled with Site-Directed Spin Labeling (SDSL) is a powerful methodology for determining long-distance (2-8 nm) structural constraints in proteins, unchallenged by conformational heterogeneity and suitable for proteins of any size or complexity[3]. As such, it is an ideal spectroscopic technique for studying the excited state ensemble populated at high-pressure.

❖Our goal for this study is to devise and evaluate a strategy for determining structural changes in pressure-induced excited states with DEER, and utilize this methodology to investigate the pressure-induced molten globule state of apomyoglobin.

Myoglobin

❖Holomyoglobin is highly ordered and practically devoid of large amplitude conformational fluctuations at neutral pH. Apomyoglobin, with the heme removed, has increased flexibility throughout the protein and local unfolding in the F helix and N-terminal end of the G helix[4,5].

❖Seven doubly-labeled mutants were generated using the nitroxide side chain R1, shown in stick representation at residue 57 to the right. The spin-labeled residues of each mutant, as well as the helices spanned, are indicated in the holomyoglobin structures to the right (PDB: 2MBW).



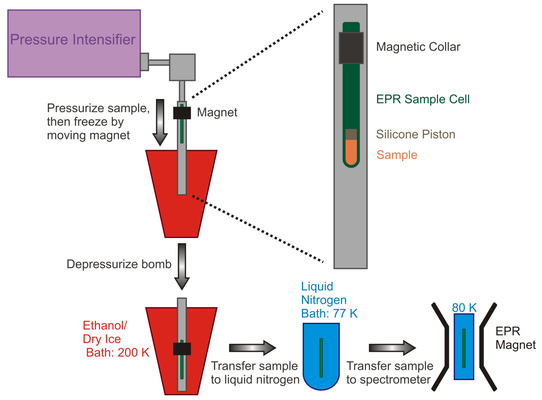
❖Apomyoglobin and holomyoglobin were prepared in the native state at pH 6.0 for this study, and DEER data will be acquired from 0 to 3000 bar.

High-pressure DEER methodology

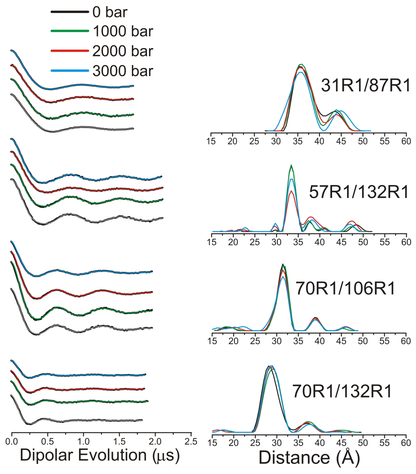
❖DEER data acquisition is done at cryogenic temperatures. Our approach is to kinetically trap the high-pressure equilibrium by freezing the sample under pressure, then depressurize the frozen sample for subsequent data acquisition at atmospheric pressure and cryogenic temperature. The procedure is shown in the diagram to the right.

❖To determine the effect of cooling to 200 K followed by cooling to 77 K, rather than cooling directly to 77 K as is typical for DEER[6], we collected data at atmospheric pressure for each mutant using each cooling method.

❖The distance distributions are nearly identical using the two cooling methods, indicating their equivalence with respect to conformational equilibria.



Holomyoglobin



❖Holomyoglobin exhibits minimal pressure response in the pressure range used in this study. The general pressure-induced change in the distance distribution is a slight broadening of the peaks already present at 0 bar., as seen in the subset of DEER data for holomyoglobin shown to the left.

❖A recent NMR study of the effect of pressure on the order parameter for side chain methyl rotation in a stable protein found that motional order increased as a function of pressure[7]. On the contrary, we find that the amplitude of motion in holomyoglobin increases slightly with pressure.

❖The response of the spin label side chain to pressure remains largely uncharacterized. As the pressure is increased, there are no significant shifts in the relative population or widths of individual peaks in the distance distributions. This supports the conclusion that pressure has no significant effect on the rotameric populations of the spin label.

Apomyoglobin

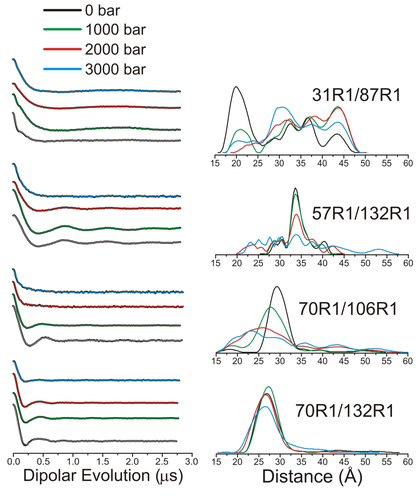
❖Pressurization of apomyoglobin leads to three general classes of response in the DEER data, illustrated by the subset of results from the 7 mutants used in this study at the right:

❖70R1/132R1: Minimal changes at high-pressure, reflecting stable helices that maintain their packing.

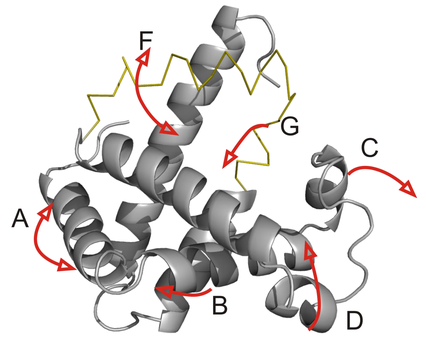
❖57R1/132R1 and 70R1/106R1: General broadening of the distance distribution.

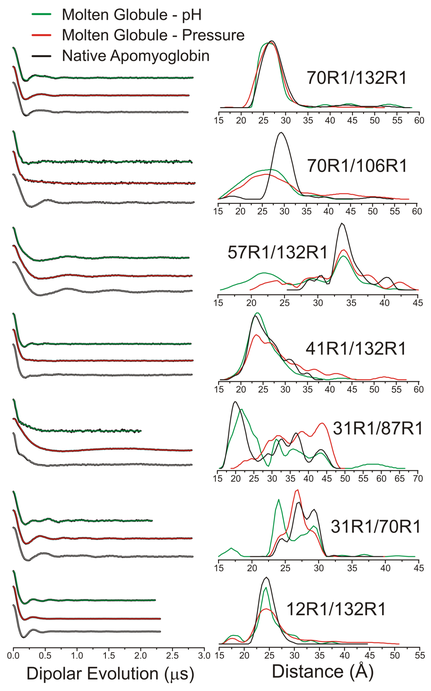
❖31R1/87R1: Shifts in the relative population of distances already present in the native state at 0 bar.

❖The large and variable changes identified in the apomyoglobin high-pressure DEER data confirms that our approach of kinetically trapping the high-pressure equilibrium is working. A pressure-induced molten globule is known to be populated at 2000 bar, and a thorough examination of this state is presented next.



High-pressure molten globule of apomyoglobin





❖Apomyoglobin is known to form a pressure-induced molten globule state at 2000 bar and pH 6.0 from solution NMR[8] and tryptophan fluorescence[9] experiments. However, severe line broadening in due to conformational exchange made structure determination by NMR impossible.

❖The characteristic timescale of EPR is nanoseconds, therefore high-pressure DEER provides a snapshot conformational exchange frozen in time. This makes high-pressure DEER an ideal approach to determining the conformational changes in the pressure-induced molten globule.

❖DEER data for native apomyoglobin at pH 6.0 and 0 bar (black), the pH-induced molten globule at pH 4.1 and 0 bar (green), and the pressure-induced molten globule at pH 6.0 and 2000 bar (red) are shown to the left.

❖Recent high-pressure circular dichroism and continuous wave (CW) EPR experiments have shown that apomyoglobin does not lose any helical structure in the transition from native state to pressure-induced molten globule. Therefore, structural changes identified by high-pressure DEER are interpreted as rigid-body motions of intact helices, rather than local unfolding, in the model of the conformational fluctuations in the pressure-induced molten globule state shown to the above left.

❖Next, we sought to compare the structure of the molten globule states populated by low pH and high-pressure. The pH-induced molten globule experiences local unfolding in the C, D, E, and F helices[10], whereas the pressure-induced molten globule maintains the helical content of the native state. Despite this, the pressure-induced molten globule exhibits a similar degree of conformational heterogeneity, as judged by the relative population of native-like distances in the molten globule state.

❖Our high-pressure DEER experiments show that this dynamic disorder doesn't correlate with large-amplitude conformational fluctuations.

Conclusions

❖The high-pressure equilibrium is kinetically trapped by freezing the protein under pressure.

❖The pressure-induced molten globule of apomyoglobin exhibits significant large-amplitude conformational fluctuations, despite maintaining native-like secondary structural content.

❖Large-amplitude structural changes do not necessarily coincide with loss of secondary structure.

❖Pressurization of a stable protein such as holomyoglobin results in an increase, not decrease, of the amplitude of local fluctuations.

❖The relative population of rotameric states of the nitroxide side chain R1 is pressure insensitive.

References

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