

# Alteration of Citrine Structure by Hydrostatic Pressure

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In 1914 Percy Bridgman, the Nobel prize winning father of high pressure physics, squeezed hen egg white at high pressure and found that it coagulated, as though it had been hard-boiled<sup>1</sup>. He had discovered that proteins denature under pressure, resulting in a coagulated state reminiscent of what happens when proteins are thermally denatured. Since Bridgman's pioneering observation many hundreds of papers have reported on the effects of high pressures on biomolecular systems: Proteins unfold, enzymatic rates change, multimeric assemblies have greatly altered stabilities, viral infectivities are greatly different, etc. Frequently, the changes are of very large magnitude and occur at pressures encountered in the biosphere. Yet proteins are very incompressible, typically 10 times stiffer than water, so the actual change in volume is very small, even at a few thousand atmospheres pressure. What are the mechanisms of the pressure effects? What do these mechanisms teach us about protein function?

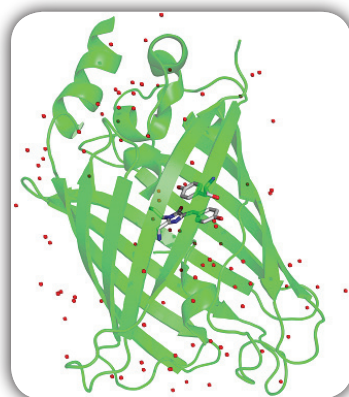
Little is known about structural changes in proteins in response to pressure and the way the resultant changes affect function. The main reason for this lack of understanding is that very few protein structures have been solved as a function of pressure. Another reason is a cultural bias that pressure effects are esoteric and have little relevance to understanding proteins. This thinking is misguided for two reasons: First, there are significant pressure effects observed in the biosphere. For example, certain essential enzymatic processes adapted for deep sea life fail at atmospheric pressure, and vice versa. Second, pressure is another thermodynamic "knob" that can be turned by the experimenter to gain insight about protein function. Put another way, the free energy that governs molecular reactions is related to  $d(PV - TS)$ , where  $P$  is pressure,  $V$  is volume,  $T$  is temperature

and  $S$  is the entropy. In many protein systems, a change in pressure of a thousand atmospheres is comparable in magnitude to a change of many tens of degrees in temperature.

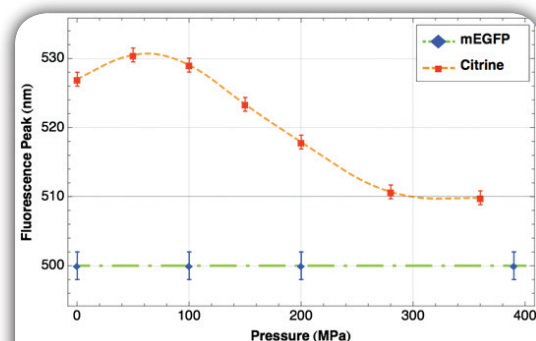
In recent years we have developed methods of data acquisition and analysis to perform protein crystallography at pressures ranging to several thousand atmospheres in order to study pressure-induced structural changes<sup>2,3,4</sup>. The studies have provided a wealth of information on phenomena, including pressure-induced protein unfolding<sup>5</sup>, water penetration into proteins<sup>4,6</sup>, and conformational changes that affect function<sup>2,7</sup>. A general theme that emerges is that pressure induces a host of structural displacements at the level of a few tenths of an angstrom. Although these displacements are well below typical resolution limits for observation of single atoms in proteins, they are readily observed for collections of protein residues and secondary structures by observing changes in the center of mass of the protein parts. This should not be surprising – recall that changes in displacements of atomic force microscope cantilevers are routinely observed at the fraction of an angstrom level, even though the resolution of the visible light optical systems involved is only a few tenths of a micron.

Why are these small changes significant? Most enzymatic reactions involve conformational changes of only a few tenths of an angstrom at active sites. Therefore, changes of this magnitude can distort active sites resulting in big effects on reaction rates. Observation of the structural changes and consequent effects on reaction rates provides important insight on the detailed structural requirements of enzyme reactive sites.

A recent study at CHESS used high pressure crystallography to understand the pressure sensitivity of fluorescence in the protein Citrine<sup>7</sup>. Citrine is a modified green fluorescent protein (GFP) whose peak fluorescent wavelength is known to shift with pressure. All proteins in the GFP family have a  $\beta$ -barrel structure with a fluorescent chromophore at the center, formed by the autocatalytic fusion of three amino acid residues (Fig. 1). In Citrine, an additional tyrosine ring is stacked upon the chromophore. The fluorescence peak of Enhanced GFP (mEGFP) is not pressure dependent to at least a few thousand atmospheres (Fig. 2) (wild type GFP does



**Fig. 1:** Ribbon diagram of Citrine showing the residues that make up the chromophore in the core of the protein<sup>7</sup>.



**Fig. 2:** Change of the peak fluorescent wavelength with pressure for Citrine (red squares) and a modified GFP (blue diamonds)<sup>7</sup>.

display pressure sensitive behavior, for reasons that are related to the reduction in fluorescence intensity of Citrine rather than the shift in fluorescence peak). Basic quantum chemistry suggests that the peak fluorescent wavelength of a stacked aromatic system would be very sensitive to the detailed coupling, e.g., the relative positions of the  $\pi$ -bonds of the aromatic rings. Since GFP has a single aromatic chromophore, it would be relatively insensitive to movements of the chromophore with pressure. On the other hand, Citrine, with a stacked aromatic chromophore, would be very sensitive to small displacements of one aromatic ring relative to the other. Our study sought to observe if there is a relative displacement of the expected magnitude with pressure. If so, this may be taken as generally analogous for what happens in active sites in enzymes. That is to say, comparable distortions of active sites can be expected to alter reaction rates, thereby explaining in a general way why enzymes are pressure sensitive.

The relative motions versus pressure of the two aromatic rings are shown in Fig. 3. What gives rise to these displacements? The two aromatic rings that make up Citrine's chromophore are connected to very different parts of the single polypeptide chain from which Citrine is folded. One of the rings hangs off the inside of the  $\beta$ -barrel while the other is in another part of the polypeptide chain that threads through the axis of the barrel. Thus, distortions of the overall structure are sensitively coupled to relative displacements between the stacked rings. Specifically, the residues that make up Citrine can be grouped into two clusters. Cluster 1 makes up one side of the barrel

and Cluster 2 includes the threading polypeptide chain. A careful analysis of the changes in overall Citrine structure with pressure shows that Cluster 1 contracts, resulting in a bending motion relative to residues of Cluster 2. The relative motions of the two clusters result in relative motions of the two rings.

This study illustrates how collective distortions of the overall structure of a protein are conveyed to critical parts of the structure necessary for "function", "function" in this case being peak fluorescent wavelength. Citrine was chosen for this study because there is good fundamental understanding of how distortions of the stacked aromatic rings should affect the peak fluorescence. In enzymes, however, the detailed structural requirements of active sites are frequently less well understood. The Citrine study also suggests how pressure studies on proteins may guide mutagenic engineering to optimize enzymatic function. Given the observed distortions of the chromophore required to achieve a given peak fluorescence shift, can these be used to guide programmed mutations to generate the shift at room pressure? The study suggests that insertion of smaller residues in the barrel side of Cluster 1 might result in the same kind of contraction needed to mimic the shift seen at high pressure. This experiment has yet to be performed, but it suggests an overall strategy to optimize enzymes<sup>7</sup>: As noted earlier, many enzymes have pressure-dependent catalytic rates. The strategy would involve observation of the structural changes that result in increased activity with pressure. Once these are identified, one would attempt

to perform targeted mutagenesis to mimic the structural deformations.

The Citrine study has shown that the pressure-dependence of protein function can be understood in terms of straightforward collective displacements of protein structure. Prediction of the exact conformational changes that occur is beyond the present state-of-the-art, but experimental observation of the changes has been demonstrated. In the future these may be used to engineer protein activity.

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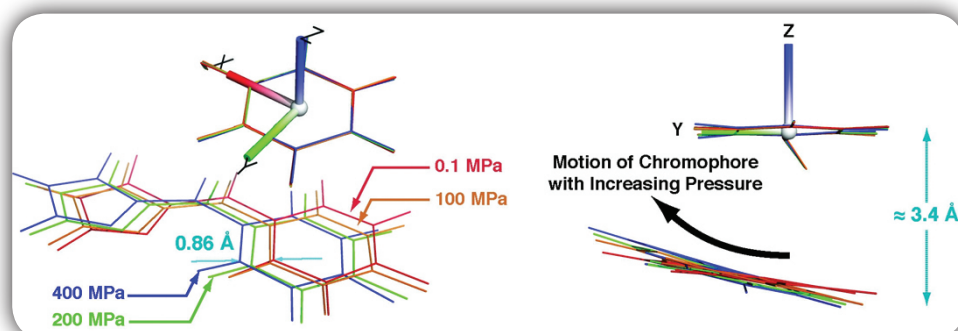


Fig. 3: Movement of one part of the chromophore relative to the other with pressure<sup>7</sup>.