HDX for Determining Order of Binding

Proteins often bind more than one ligand and the order in which these ligands binds can affect protein function (e.g., allostery). This binding order can be used to guide research on drug development, particularly when the protein-ligand system is asymmetrical (i.e., different ligand binding regions are not identical). One example is aminoglycoside-*N3*-acetyltransferase-IIIb (AAC) (1), whose order of substrate binding (either coenzyme A or antibiotic) yields ternary complexes with different dynamic properties.

An approach based on PLIMSTEX has been developed to provide this information (2) and has been applied to calcium binding in troponin, a protein which binds four Ca^{2+} ions in four EF hands. The affinity of these sites vary widely so a two-step dialysis procedure was carried out to ensure a that the starting point had no bound Ca^{2+} (i.e., it was a very pure apo state). In the work flow, a "sharp break" PLIMSTEX curve is first obtained at high protein concentration to reveal the binding stoichiometry, and give an estimation of the ΔD values to guide the curve fitting process that provides equilibrium constants. Fractional species curves are generated from these binding constants and the concentrations of the bound species are determined, that is, troponin with between 0 and 4 bound Ca^{2+}. Continuous labeling using various concentrations of Ca^{2+}, allows the determination of which regions bind Ca^{2+} at each concentration since HDX reports changes caused by ligand binding at each site. For troponin the determined binding order to four EF hands is III, IV, II and I (Figure 1).

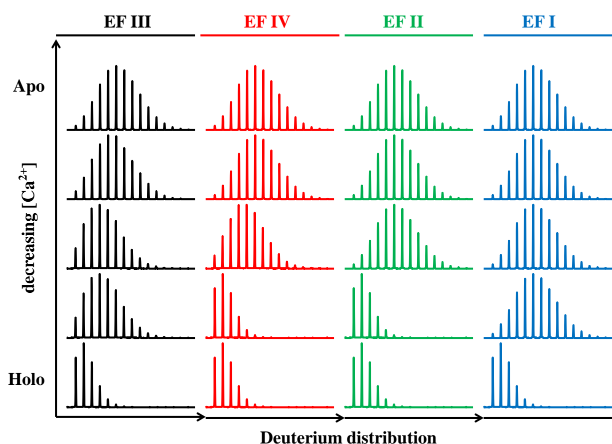

Figure 1. HDX patterns for various Ca^{2+} binding sites (obtained as peptides by peptic digestion) indicated the binding orders. Each column represents deuterium distribution of the peptic peptides for each EF hand: III, IV, II, and I (left to right). Each row represents various Ca^{2+} bound states: 0, 1, 2, 3, and 4 Ca^{2+} bound (top to

One advantage of identifying binding order using this HDX method is that the measurement relies on mass shifts that can be measured with great accuracy and sensitivity using mass spectrometry rather than trying to directly measuring concentration of each species in a complex mixture. The equilibrium information obtained from the PLIMSTEX experiment is essential for the design of the subsequent kinetics experiments that extract deuterium distribution for different binding regions. For a tight-binding system, no binding order information can be obtained. Compared to traditional approaches, this method is time-consuming. Nevertheless, this method can be applied to proteins that bind multiple ligands, without disturbing the system by mutating the protein. In addition, it can be carried out in more complex systems and in the presence of other solvent molecules.

1. Norris, A. L., Nickels, J. D., Sokolov, A. P., and Serpersu, E. H. (2013) Protein dynamics are influenced by the order of ligand binding to an antibiotic resistance enzyme, *Biochemistry*.
2. Huang, R. Y., Rempel, D. L., and Gross, M. L. (2011) HD exchange and PLIMSTEX determine the affinities and order of binding of Ca2+ with troponin C, *Biochemistry 50*, 5426-5435.