HDX for Measuring Affinity

PLIMSTEX

To measure protein/ligand affinities, we developed PLIMSTEX (1, 2) and in an early application, used it to measure the interaction of human telomeric repeat binding factor 2 (hTRF2) with telomeric DNA (3). The approach is unique because is measures not only the HDX of bound and unbound protein, but also that of intermediate mixtures of apo-protein with ligand. The approach is to conduct a titration, generating a PLIMSTEX curve. This curve can be fit with a 1:n (protein:n ligands) sequential binding model to extract the binding constants. In addition to analyzing the global HDX, pepsin digestion can be used to produce constituent peptides whose extent of HDX serve to localize regions where exchange occurs.

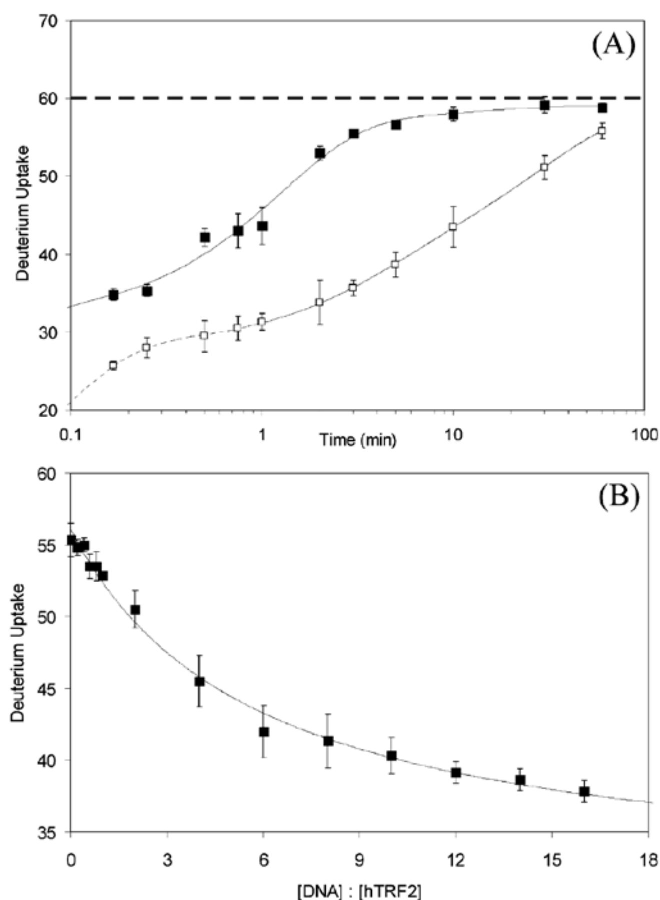

Figure 2(A). Global deuterium uptake kinetics over 60 min for apo (■) and holo (□) hTRF2 (1 M). (B) PLIMSTEX titration curve obtained by monitoring the deuterium uptake of hTRF2 (100 nM) upon adding increasing amounts of the ODN ligand.

The first step in a PLIMSTEX determination is to acquire the rate at which the apo- and holo-forms of the protein gain deuterium. For the hTRF2-DNA example, the apoprotein exchanges roughly the same number of amides at long time points, but the rate of exchange is much faster than that of the holoprotein (Figure 2A). The time at which the difference in deuterium (ΔD) is the greatest and approximately steady-state is chosen for the PLIMSTEX titration. In this step, the ligand is added to the protein in increasing concentrations from zero to excess. After the solution has reached equilibration, a deuterated buffer is added so that the final concentration has a reasonable concentration of D_2O (e.g., 95% deuterium although lower percentages can be used). The exchange is “quenched” at the time specified in the kinetics experiment (here 3 min). After measuring the extent of HDX with a mass spectrometer, one obtains a curve showing the HDX extent in going from the apo to holo form (Figure 2B). Mathematical modeling (4) reveals the binding constant; in this example, the K_d for hTRF2-DNA is 580 $\pm$ 140 nM, in good agreement with that determined previously by surface plasmon resonance.

Although the global exchange data are useful for characterizing the protein-DNA complex and determining affinity, exchange at regions of the protein or even at the amino-acid level can reveal binding regions and perhaps report on allosteary. As is the case with other HDX approaches, enzymatic digestion of the exchanged protein followed by MS analysis of the resulting peptides provides this specificity. Owing to the small size of hTRF2, only five peptic fragments are needed to give complete coverage. Peptides located in the unstructured region are highly deuterated, and the ΔD is small. Those in the binding region show a large ΔD; the holo exchanged far less than the apo. This is one example showing that PLIMSTEX provides a reliable means of determining affinity at both the global and the peptide level of a protein. Determinations at the peptide or regional level is the topic of ongoing research at the resource, and we welcome collaborations.

dPLIMSTEX

An obstacle to extending PLIMSTEX to large proteins (e.g., antibodies) is that current PLIMSTEX consumes relatively large amounts of protein (e.g., µg). Another obstacle is deuterium uptake of the antibody upon ligand binding will be a small fraction of the total number of exchangeable amides, making accurate measurement difficult. To overcome these obstacles, we developed a modified PLIMSTEX method that incorporates a dilution strategy to complement the titration and monitor the HDX of the smaller protein or peptide (5). To conduct this experiment, we begin with a solution of protein and ligand or of two proteins whose interaction is of interest. For each ligand/protein ratio, we will allow equilibrium to be established for some fraction (e.g., 1/3) of the volume. Another 1/3 aliquot will be diluted by a factor of 2 and also equilibrated, and the last 1/3 aliquot will be diluted by a factor of 4 and equilibrated. After equilibration, each aliquot will be mixed with an equal volume of D_2O buffer and allowed to undergo HDX, followed by quench and LC/MS analysis. In this way, we conserve most of the original protein while obtaining multiple curves that are fitted simultaneously. The fitting gives the K_a. An example of d-PLIMSTEX curves is in Figure 3.

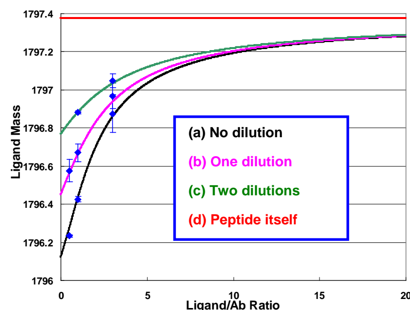

Figure 3. Example d-PLIMSTEX curves: (a) is at the highest conc; (b) for one dilution, (c) for two dilutions, and (d) the peptide ligand and asymptote of all HDX curves.

SIMSTEX

To study the self-association properties of proteins by ESI-MS and HDX, one can take advantage that differences in the HDX of monomers and oligomers are different. For example, recombinant human insulin (6) shows significantly more protection for in HDX compared to lispro insulin, which may be due to differences in self-association

We adapted PLIMSTEX and its modeling to determine self-association equilibrium constants for proteins and applied it to various insulin analogs (7); we term this adaptation SIMSTEX (Self Interactions by Mass Spectrometry self Titration and HD EXchange). From the method comes affinity constants, which compare well with the literature results. The results from SIMSTEX show that some mutants (e.g., GlnB13) of insulin have an increased tendency to self associate, possibly slowing down their action in vivo. Other mutants (e.g., lispro and AspB9) have lower propensities for self-association, thus providing potentially faster-acting analogs for use in controlling diabetes. As in PLIMSTEX, the shape of the curve contains information on the stoichiometry and binding constants for this small protein-protein interaction (Figure 4). We are interested in applying this approach to other oligomerizing proteins.

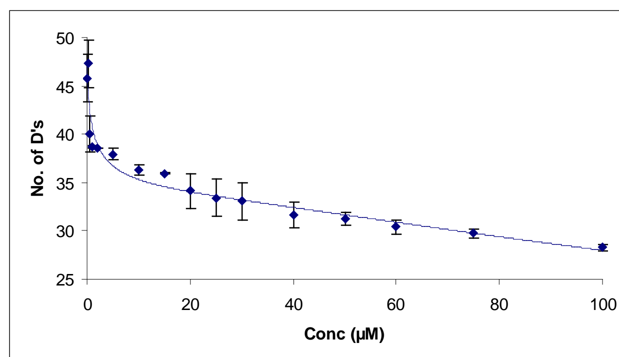

Figure 4. SIMSTEX plot of extent of HDX as function of solution concentration of r-Human insulin. Points are experimental, and the curve is the theoretical fit.

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