

# ExSAR<sup>TM</sup>

## ***PROTEIN-PROTEIN INTERACTION CHARACTERIZATION BY HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY***

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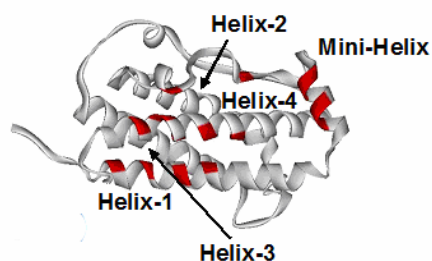
# OVERVIEW

## PROTEIN-PROTEIN INTERACTION STUDIES

Previously, a high affinity variant of human growth hormone (hGHv) was generated by phage display mutagenesis (Lowman and Wells, 1993). This variant bound the extracellular domain of its cognate receptor (hGHbp) ~400 times greater than wild-type human growth hormone (hGHwt) (Pal et al., 2003).

Within this high affinity variant were found fifteen mutations corresponding to the binding interface (see Figure 1). This made for a more hydrophobic surface and an altered intramolecular H-bonding network with hGHbp. Surprisingly the binding energy predicted from these new interactions failed to account for the large increase in affinity (Pal et al., 2003). Professor Anthony Kossiakoff from the University of Chicago contracted ExSAR's H/D-Ex services to probe the protein dynamics of hGH and hGHbp in order to investigate the extent to which protein dynamics influenced the association of these two biomolecules (Horn et al., 2006). The purpose of this overview is to briefly illustrate how protein dynamics can influence a protein-protein interaction and how H/D-Ex provided the necessary means to reach this conclusion.

### Mutation Sites



**Figure 1.** The 15 mutated residues are red highlighted in the ribbon diagram of hGHv (PDB Identifier 1HUW). These substitutions correspond to F10A, M14W, H18D, H21N, K41I, Y42H, L45W, Q46W, F54P, R64K, R167N, D171S, E174S, F176Y, and I179T. Helices 1-4 and the mini-helix which form the hGHbp binding surface are indicated in bold text.

### I. Analysis of Protein Dynamics by H/D-Ex

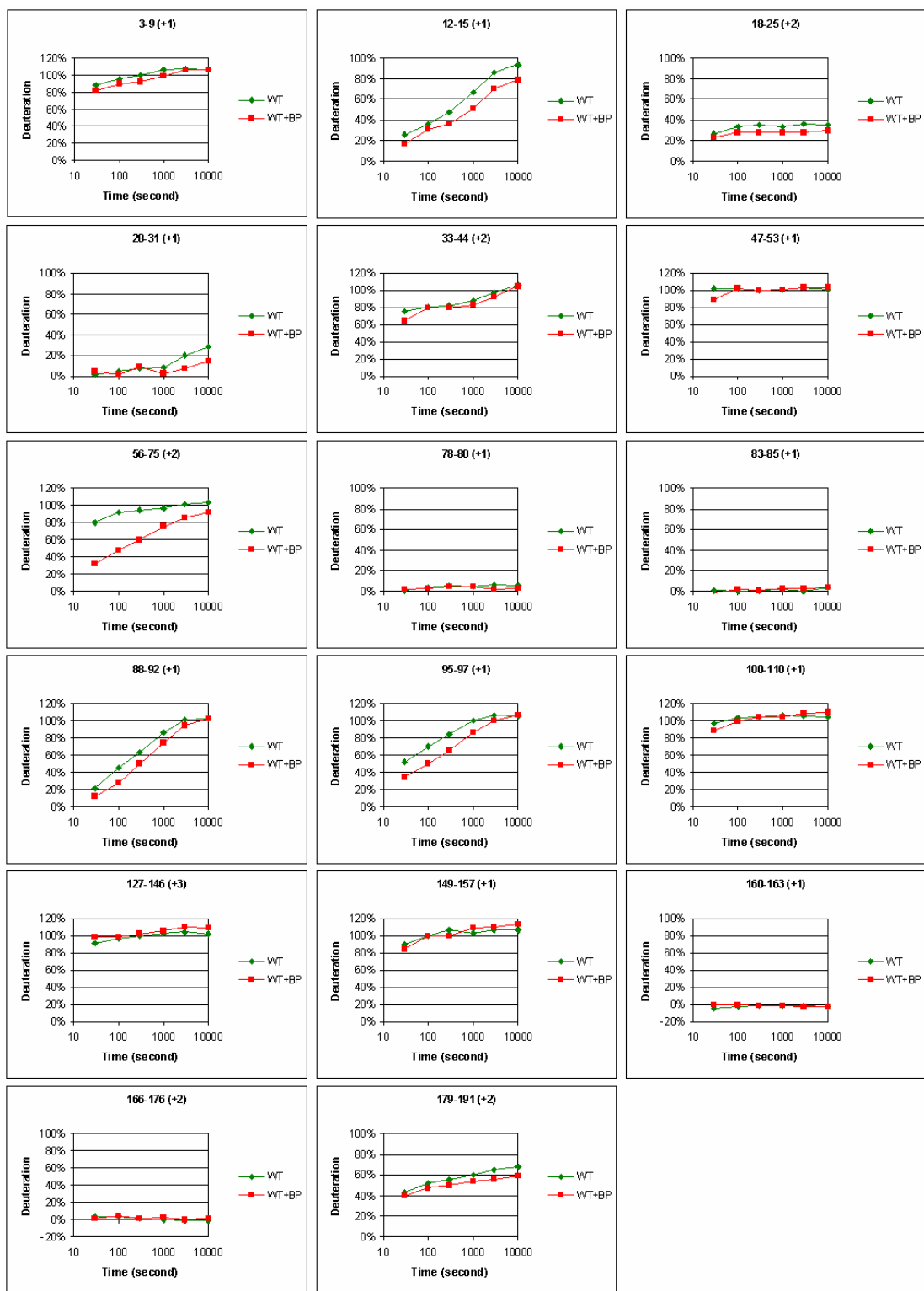
The protein dynamics of free and hGHbp associated hGHv and hGHwt were probed by ExSAR's H/D-Ex technology. This analysis was repeated upon free and hGHv or hGHwt associated hGHbp. A schema of the technique is illustrated in section II.

Sequence coverage and resolution:

Approximately 90% of hGHv and hGHwt protein sequences were correlated to one of seventeen hGH pepsin derived peptides. 100% of the hGHbp protein sequence was correlated to one of twenty-one pepsin derived peptides. In general, peptides under analysis were of a median length of 9 residues.

Experimental Results:

In Figure 2 are illustrated a series of panels depicting the deuterium buildup of 17 peptides derived from hGHwt, both in the presence and absence of hGHbp. Similar graphs were generated for hGHv (+/- hGHbp) and for hGHbp (+/- either hGHwt or hGHv). Average differences in deuteration were calculated for corresponding peptides. For example hGHwt sequence segment 56-75 was 29% less deuterated on average than the same segment in the presence of hGHbp. This difference in deuteration is referred to as a perturbation. Negative perturbations are indicative of a slowing down of naturally occurring "breathing-like" motions or structural motions of greater magnitude.

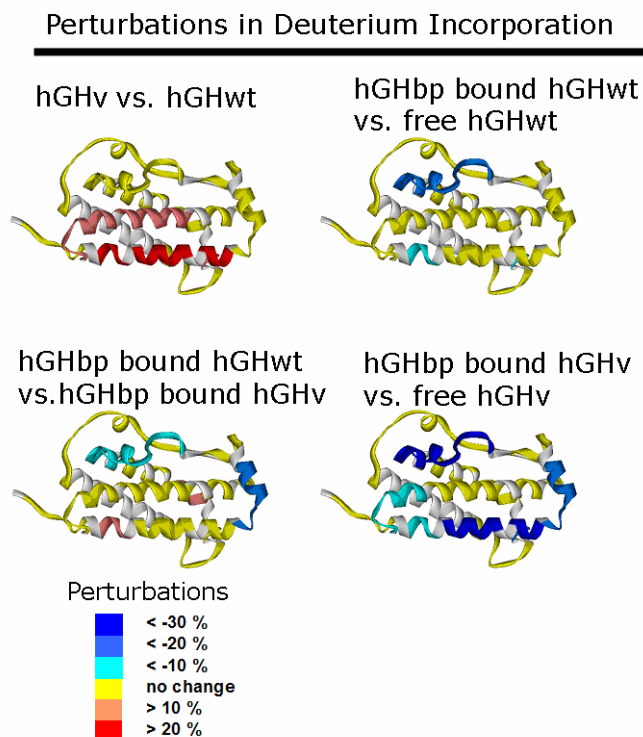


**Figure 2.** H/D-Ex results of hGHwt with (red plot) or without hGHbp (green plot). Each panel represents a segment of the protein. The residue numbers in each segment are indicated in the title and in parenthesis, the charge state of the peptide.

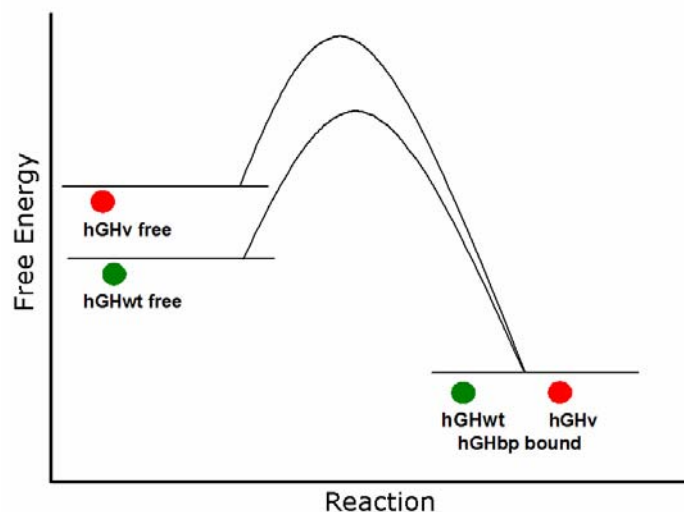
To better conceptualize the significance of a set of perturbations, the perturbation is color coded by magnitude and mapped to the crystallographic or solution structure of the protein as illustrated in Figures 3.

From Figure 3 we see that hGHv is considerably more dynamic in the vicinity of the hGHbp binding interface than hGHwt. Why this contributed to tighter hGHbp binding was not immediately apparent. As indicated in the lower left of Figure 3, the deuteration levels of hGHbp bound forms of hGHwt and hGHv were nearly identical. A significant degree of stabilization in helix-1 and the mini-helix (circled regions) of hGHv was observed upon binding hGHbp, so although hGHbp bound forms of hGHv and hGHwt possess similar dynamics, hGHv upon binding hGHbp underwent significant stabilization.

Figure 4 depicts a thermodynamic mechanism explaining the increase in binding affinity. In short, destabilization leads to energetically unfavorable interactions (Horn et al., 2003) , thus an increase in the free energy of the system with respect to non-bound hGHv. Since affinity can be correlated to the difference in free energy upon binding, a larger difference (hence greater affinity) would result from mutations that destabilize the unbound state and/or stabilize the complex. Note that protein engineering methods to date focus on the complex side of the reaction.

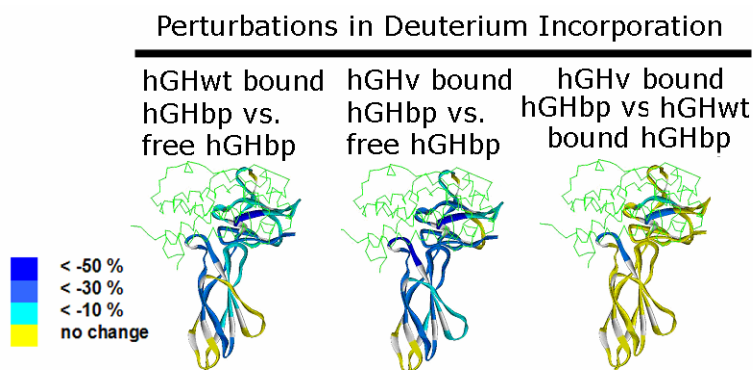


**Figure 3.** Ribbon diagram of hGHv displaying either increases in H/D-exchange (red color) or decreases in H/D-exchange (blue color).

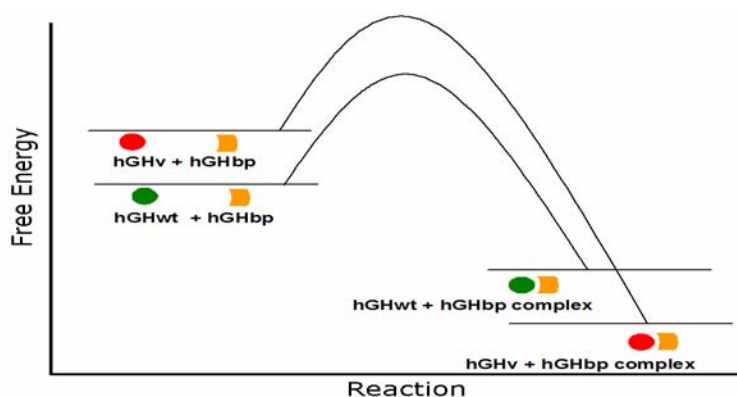


**Figure 4.** Free energy reaction diagram for hGHv or hGHwt association with hGHbp.

Differences in deuterium incorporation of free and hormone bound hGHbp were determined as previously described. These differences were color coded by magnitude and plotted on the crystallographic structure of the hGHv:hGHbp complex (see Figure 5). The reduction in exchange at the interface of hGHv bound hGHbp (circled region) is indicative of a reduction in dynamics hence greater stability in the presence of hGHv. This effectively creates a greater difference in free energy upon binding as illustrated in Figure 6. The increase in binding-affinity of the hGHv is therefore likely the result of two mechanisms, complex stabilization (hGHbp stabilization in complex) and destabilization of the unfolded state of the hormone.



**Figure 5.** Ribbon diagram of hGHbp in complex with hGHv (backbone trace) displaying various levels of decreasing exchange upon complex formation.

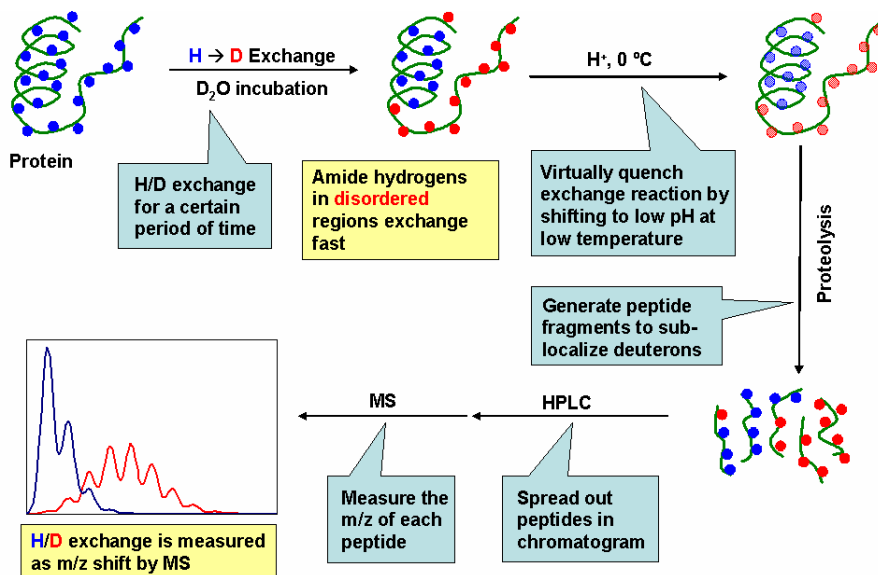


**Figure 6.** Free energy reaction diagram for hGHv + hGHbp and complex.

## II. Hydrogen/Deuterium Exchange Mass Spectrometry (H/D-Ex)

ExSAR's platform technology, Hydrogen/Deuterium Exchange Mass Spectrometry, can be used to probe the conformational dynamics of a protein's 3D structure in solution (Figure 7).

In brief, protein is mixed with deuterated buffer and incubated for a predetermined duration during which backbone amide hydrogens gradually exchange with bulk solvent deuterons. The exchange rate of each backbone amide hydrogen is unique to its environment; disordered regions and/or surface exposed regions exchange fast, ordered and/or buried regions exchange slow. Following the incubation period, the exchange reaction is essentially quenched by shifting the pH to around 2 while lowering the temperature to near 0°C. The exchanged protein is then proteolyzed with acid stable proteases. The peptic fragments are then chromatographically separated and their masses determined by mass spectrometry. The experiment is repeated in the absence of deuterium and the molecular weight difference of identical fragments attributed to deuteration.



**Figure 7.** H/D-Ex Experiment Overview

### **III. ExSAR Competencies and Deliverables**

ExSAR has offered H/D-Ex services since 2002 for major pharmaceutical and biopharmaceutical clients. It offers a collaborative and consultative approach, with verbal and written presentation of results. Its scientific leadership includes Dr. Charles Cantor, Dr. William DeGrado, and Dr. S. Walter Englander, each members of the National Academy of Science. Projects are conducted under the supervision of Dr. Yoshitomo Hamuro.

#### **William F. DeGrado, Ph.D.-Chairman of the ExSAR Scientific Advisory Board**

Dr. DeGrado is the George W. Raiziss Professor of Biochemistry and Biophysics at the University of Pennsylvania and he is a member of the National Academy of Sciences. His published research includes contributions to the fields of protein design, synthesis of peptidomimetics, and characterization of membrane-active peptides.

#### **S. Walter Englander, Ph.D. – Member, ExSAR Scientific Advisory Board**

Dr. Englander is the Jacob Gershon-Cohen Professor of Medical Science and Professor of Biochemistry and Biophysics at the University of Pennsylvania. He is a member of the National Academy of Sciences, Honorary Fellow of the Biophysical Society and Honorary Fellow of the American Association for the Advancement of Science. His work has focused on internal protein motions and correlations to amide hydrogen exchange rates.

#### **Charles R. Cantor, Ph.D. - Director, ExSAR Board of Directors**

Dr. Cantor is the Chief Scientific Officer and Chairman of Sequenom, and a member of the National Academy of Sciences. He was previously the chair and professor of the department of biomedical engineering and biophysics, and director of the Center for Advanced Biotechnology, at Boston University. Prior to this, Dr. Cantor held faculty positions at Columbia University. He was also director of the Human Genome Center Project of the Department of Energy at Lawrence Berkeley Laboratory. Dr. Cantor has published more than 325 peer reviewed articles and has been granted 26 U.S. patents.

#### **Yoshitomo Hamuro, Ph.D.—Senior Director of Technology Development**

Dr. Hamuro has led the development of hydrogen/deuterium exchange mass spectrometry analysis of protein dynamics, protein-ligand interactions and protein-protein interactions at ExSAR since joining in 2002. Prior to joining ExSAR, he was instrumental in the development of modern H/D-Ex technology at the University of California, San Diego, in the laboratory of Professor Virgil Woods. Dr. Hamuro conducted postdoctoral research on combinatorial chemistry and solid-phase chemistry at DuPont and later on antibacterial  $\beta$ -peptides at the University of Pennsylvania under Professor William DeGrado. He obtained his Ph.D. in 1996 from the University of Pittsburgh on protein structure mimetics.

#### **IV. References**

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