

Protein Conformational Changes During Protein-Carbohydrate Binding Studied by Hydrogen/Deuterium Exchange Mass Spectrometry

Daniel King, Carl Bergmann, and Ron Orlando

Department of Chemistry, Complex Carbohydrate Research Center, UGA, Athens GA 30602

Overview

- ▶ Locate Carbohydrate Binding Site
 - Look for reduced levels of Deuterium
- ▶ Observe Conformational Changes
 - Look for increased levels of Deuterium
 - Infer mechanistic processes
- ▶ Observe the Effect of Inhibitor binding
 - Look for changes in the Deuterium patterns
 - Locate binding site from protected proteolysis sites

Introduction

- ▶ A major goal of plant pathogenesis research is the thorough characterization and understanding of the interactions between pathogen derived plant cell wall degrading enzymes and plant cell wall carbohydrate substrates. Endopolygalacturonases (EPG) are a major component of the pectin-degrading activity of phytopathogenic fungi and are among the first degradative enzymes to be secreted upon fungal infection (1,2). During pathogenesis there is a potential for interaction between EPGs and plant cell wall derived EPG inhibitors known as polygalacturonase inhibiting proteins (PGIPs) (3,4). PGIPs are soluble, leucine rich repeat (LRR) glycoproteins, found in the cell wall (5). PGIPs form high-affinity complexes with EPGs in a reversible, stoichiometric manner.
- ▶ The degradation of the plant cell walls plays a significant role in both the ripening and rotting of fruits and vegetables. Agricultural communities, interested in prolonging the lifetime of crops, are concerned about inhibiting EPG activity (6). On the other hand, there are many industrial uses for an enzyme capable of degrading plant cell walls. For example, EPGs are used in the clarification of fruit juices, the removal of color from paper, and in detergents to improve the removal of stains (7,8).

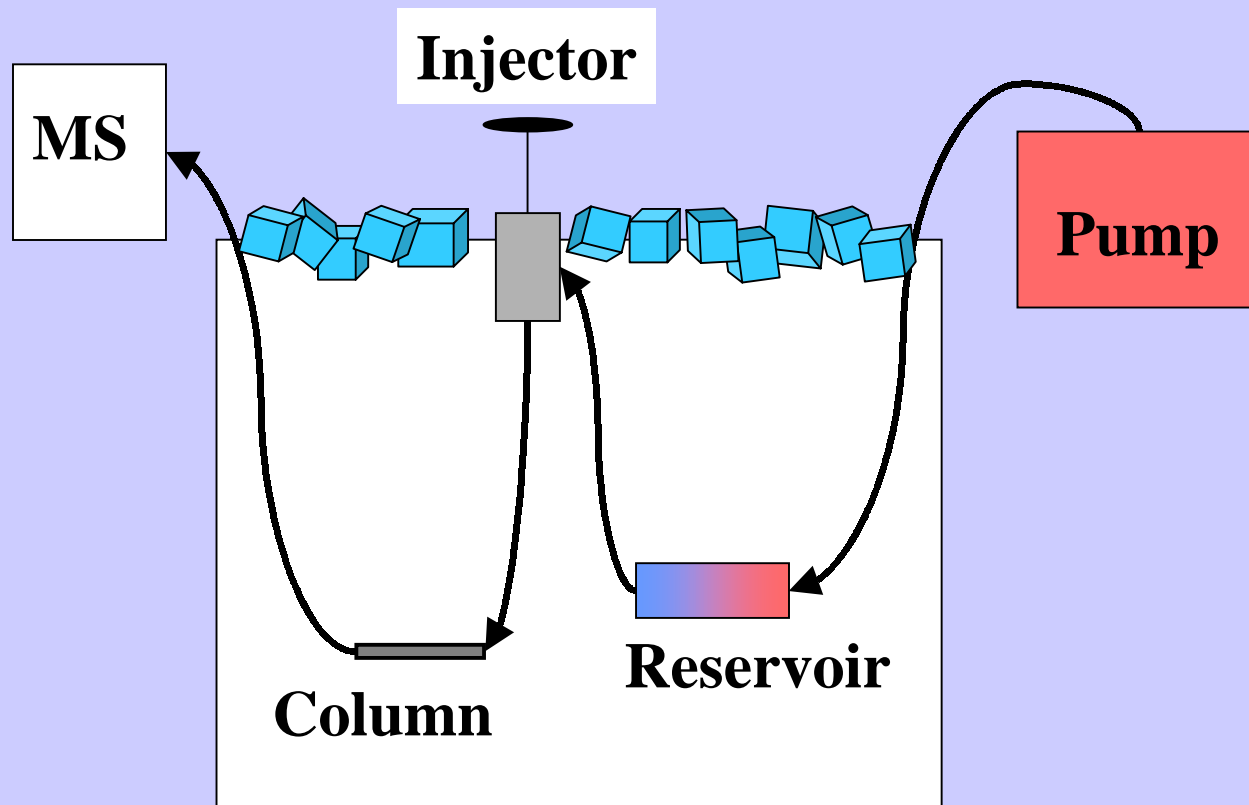
- ▶ Here we present our study of the EPG/homogalacturonan/PGIP interaction with amide hydrogen/deuterium exchange (amide exchange-MS). Amide hydrogens are labile and will freely exchange with the protons in solution if they are on the exterior of the protein, accessible to the solvent. When a protein is immersed in D₂O or a mixture of D₂O and H₂O, amide hydrogens will be replaced by deuterons, each resulting in a mass increase of 1 Dalton, a change easily monitored by mass spectrometry (9-11).
- ▶ In a recent variation of amide exchange-MS, the protein is enzymatically digested to determine specifically where deuterium is being incorporated (12-14). The deuterium exchange must therefore be quenched to prevent both new deuterium from being added to interior amino acids exposed as a result of digestion and back-exchange of the incorporated deuterium during the LC-MS process. The amide hydrogen/deuterium exchange rate decreases with temperature and pH (minimum at ~2.5). By placing the system in an ice bath and lowering the pH to 2.5, the half-life time of the deuterium on the protein can be extended to 40-50 minutes (15). Pepsin is used for proteolytic digestion because its optimal activity is at low pH. To study protein-substrate binding, a protein is analyzed in the presence and absence of substrate. The substrate will protect exterior amino acids from deuterium incorporation in the region of its interaction. This approach has been used previously to investigate sites of protein-protein and protein-ligand interactions (12-14,16).

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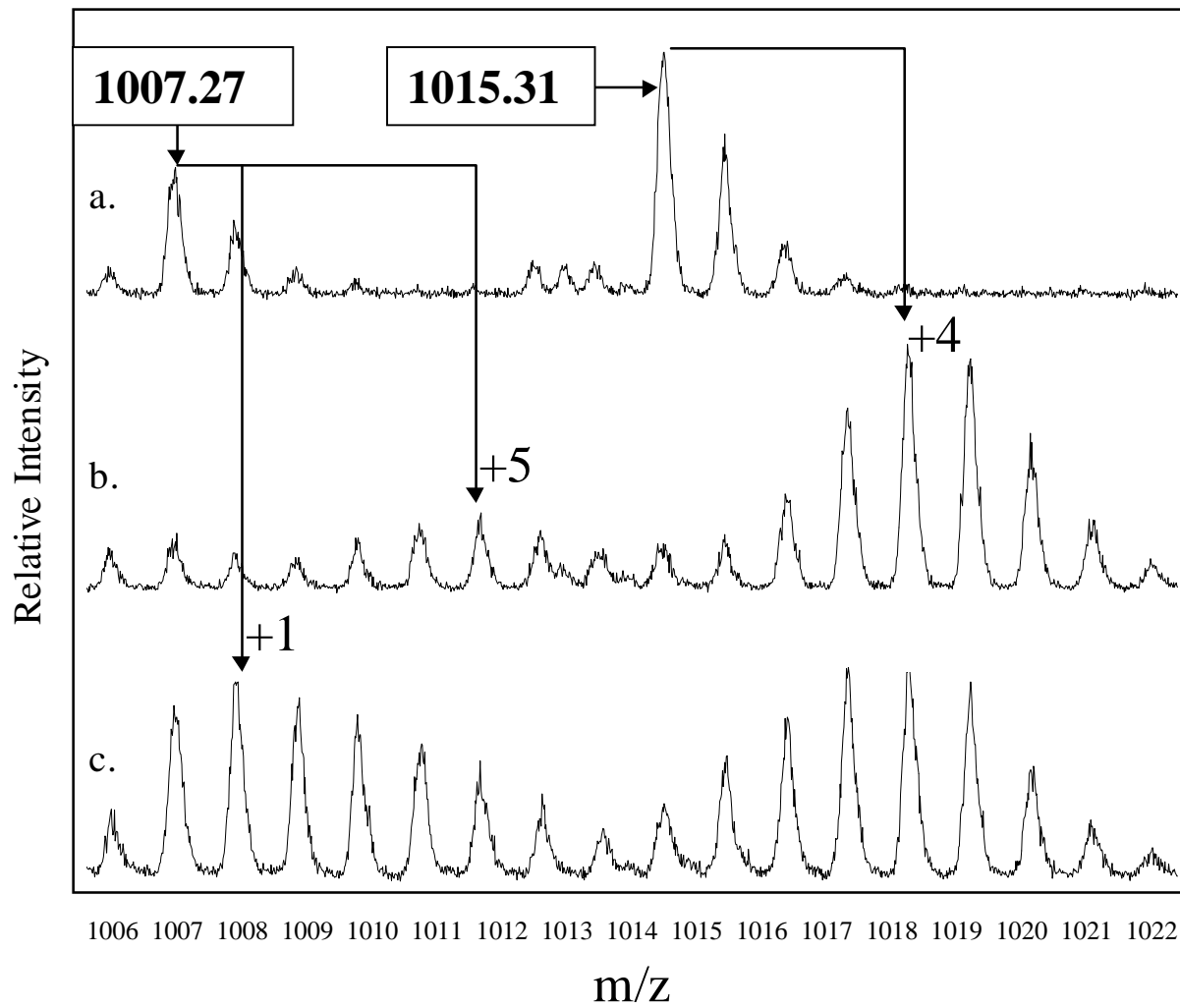
Methods

- ▶ Carbohydrate Binding - Carbohydrate (8-mer) was allowed to bind with EPG-II for 24 hours.
- ▶ H/D exchange - 10 μ g of EPG-II is solvated in H₂O or D₂O and exchanged for 24 hrs.
- ▶ Quenching and digestion - The exchange is placed into the ice bath and 0.1M HCl is added to lower the pH to ~2.5. 10 μ g of pepsin solution is added and the digestion is proceeded for 10min.
- ▶ LC separation - A linear gradient is used from 0%B to 80%B over 10 minutes with a C-18 Magic Bullet column



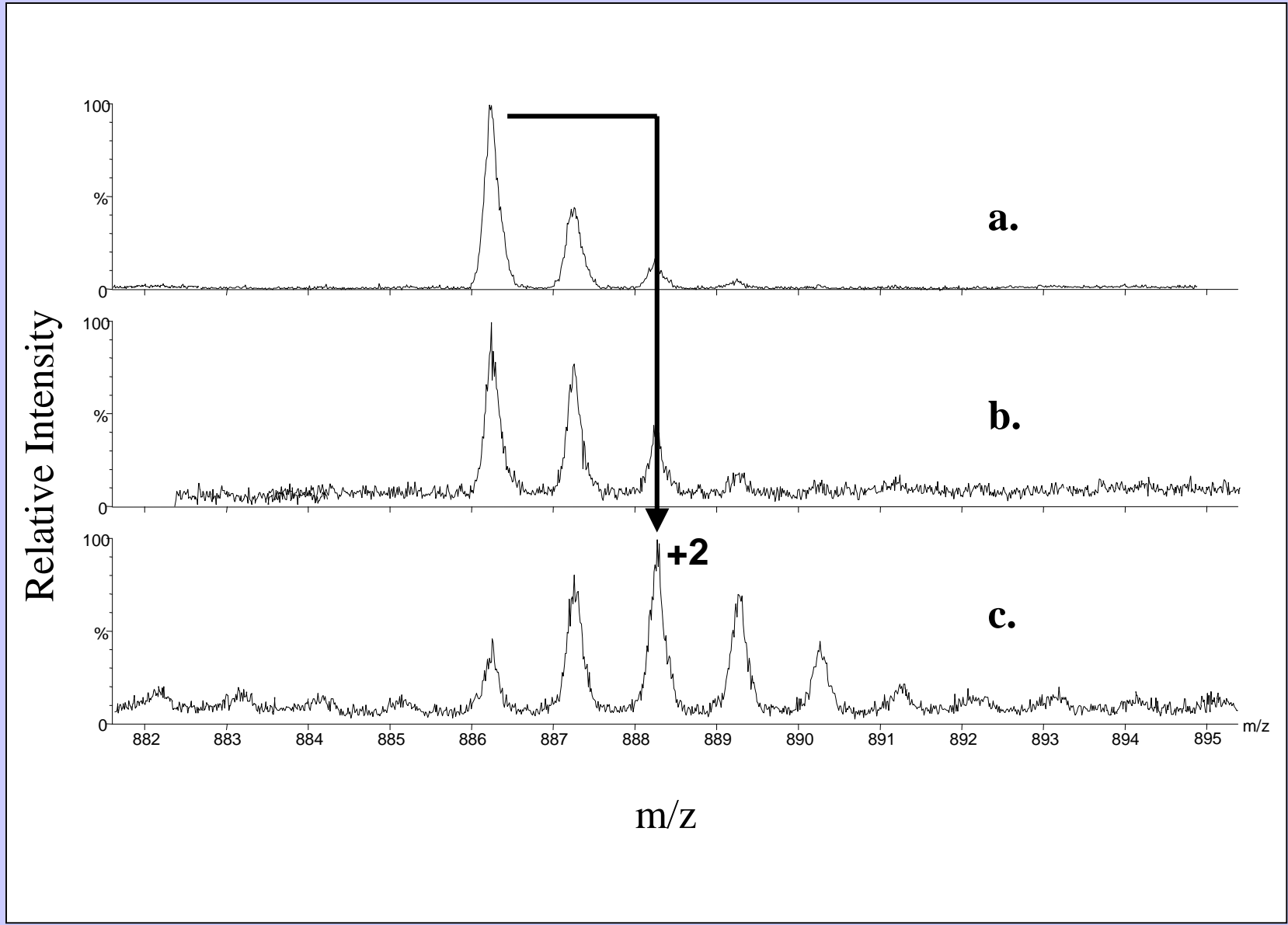
Protection

- ▶ Comparison of mass spectra
 - a.) free EPG-II in H₂O
 - b.) free EPG-II in 50% D₂O
 - c.) EPG-II/(GalA)₈ complex in 50% D₂O.
- The arrows indicate shifts in the most abundant isotope peak as a result of deuterium incorporation.



Exposure

- ▶ Mass spectra of a peptide (residues 131-139) from EPG-II
 - a.) free EPG-II in H₂O
 - b.) free EPG-II in 50% D₂O
 - c.) EPG-II/(GalA)₈ complex in 50% D₂O.
- The arrows indicate shifts in the most abundant isotope peak as a result of deuterium incorporation.



Results

- ▶ % deuterium incorporation of EPG-II
 - Estimated by dividing the number of incorporated deuterons by the number of amino acids in the peptide and multiplying by 100.
 - ▶ Changes in % deuterium incorporation
 - a.) caused by the presence of (GalA)₈
 - b.) caused by the presence of both (GalA)₈ and PGIP.
- * indicate regions in the binding cleft that are protected by the substrate.

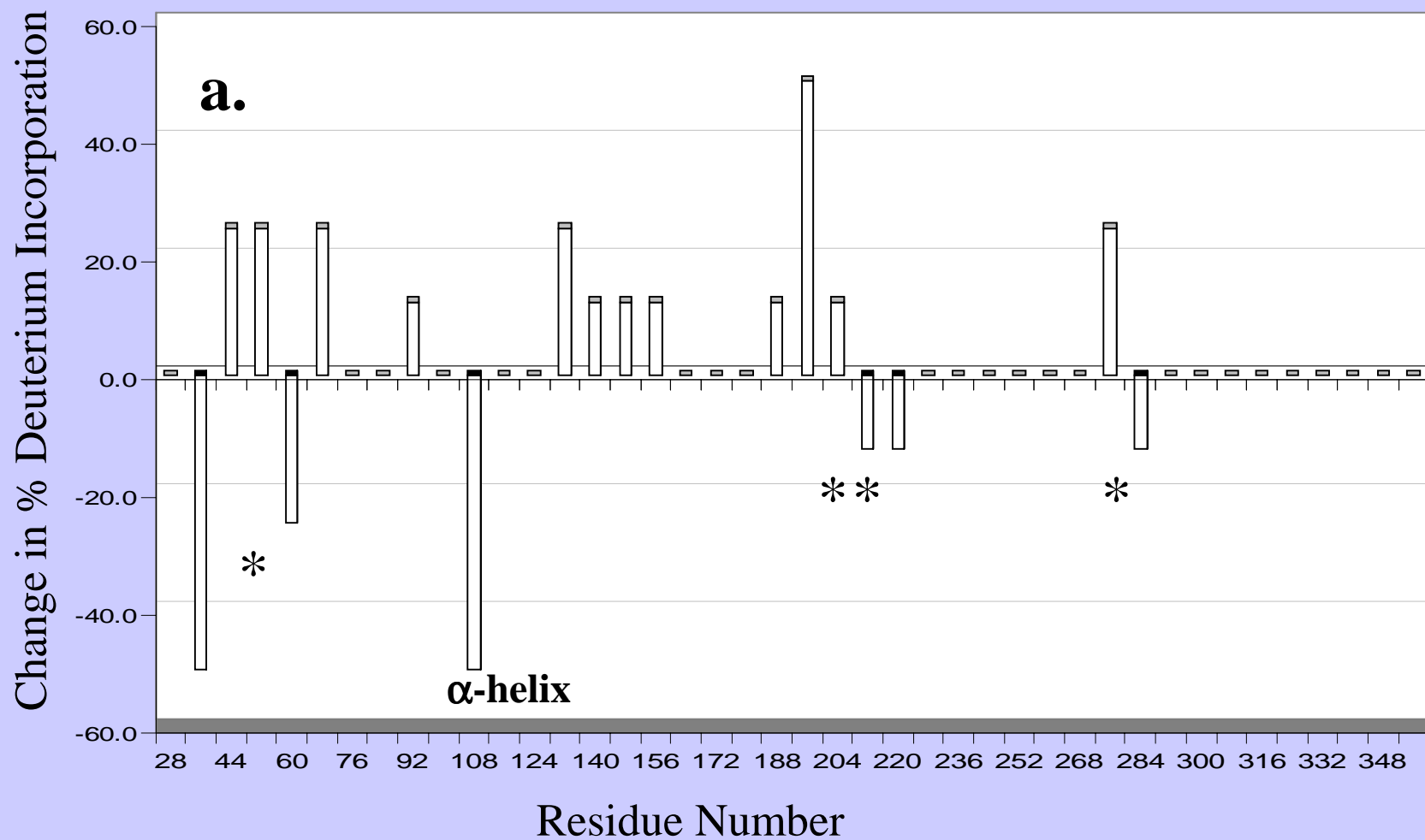
▶ Exposure (upward bars)

- Exposure occurs within the β -sheets along the underside of the barrel.
- The inhibitor eliminated or greatly reduced exposures.

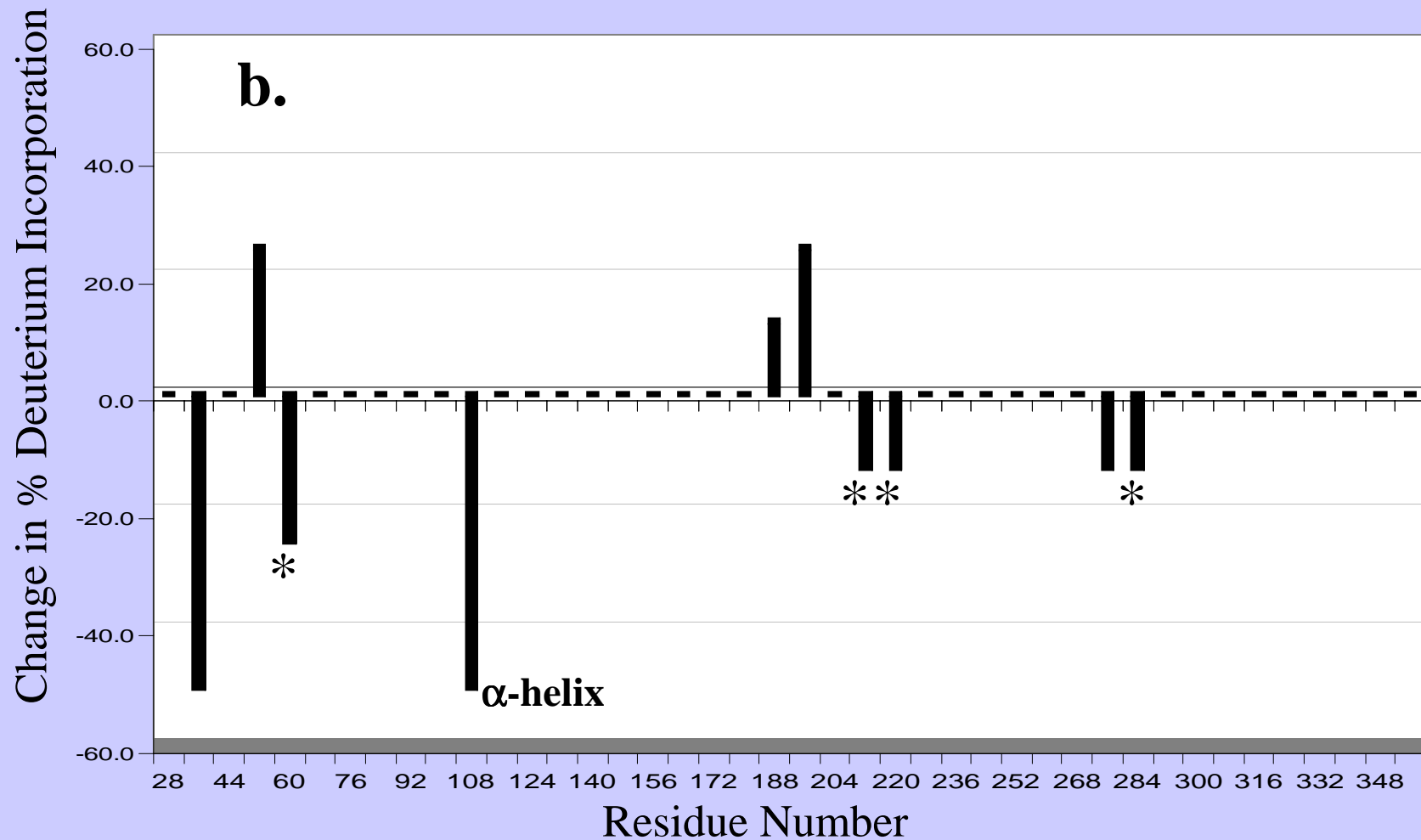
▶ Protection (downward bars)

- Binding cleft
 - * denotes protection located within the binding cleft
 - Inhibitor does not effect this protection

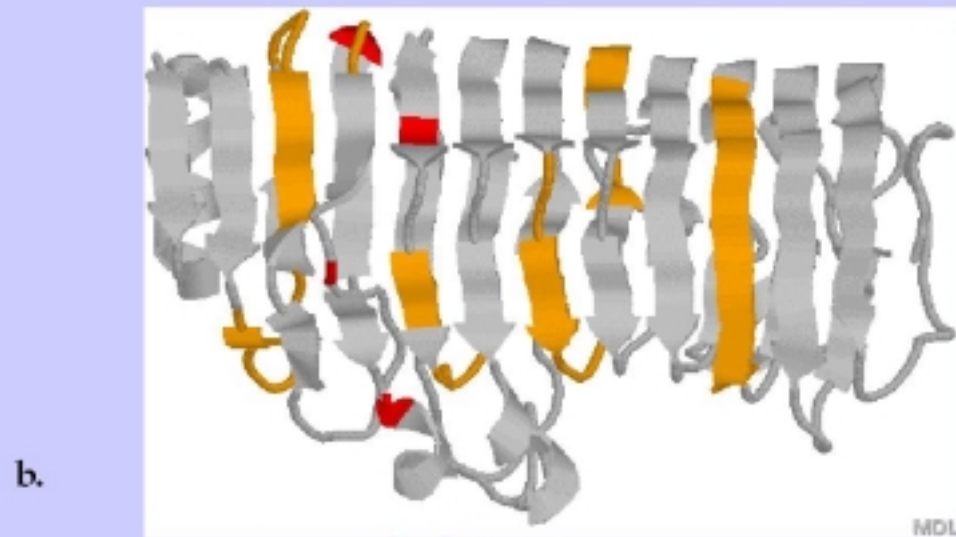
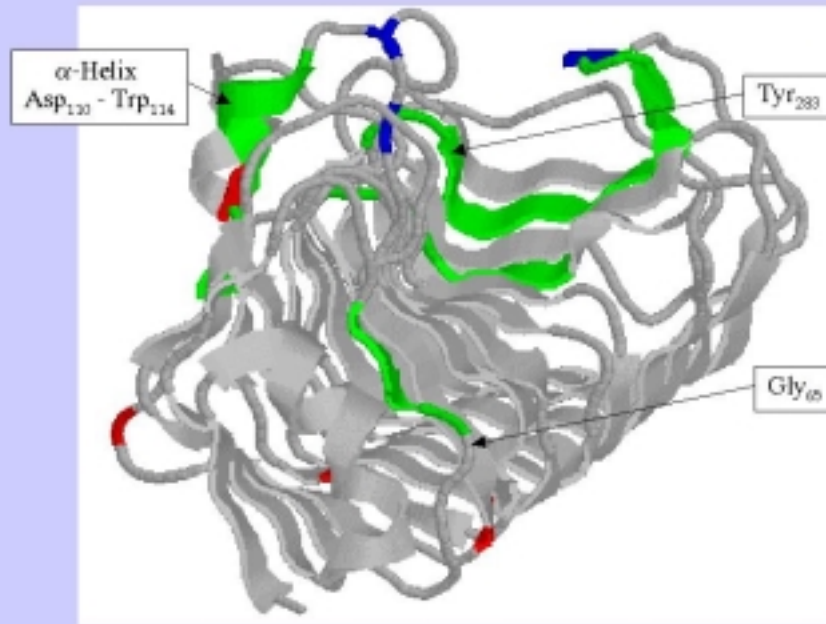
Change in % Deuterium Incorporation in Presence of (GalA)₈



Change in % Deuterium Incorporation in Presence of PGIP and (GalA)₈



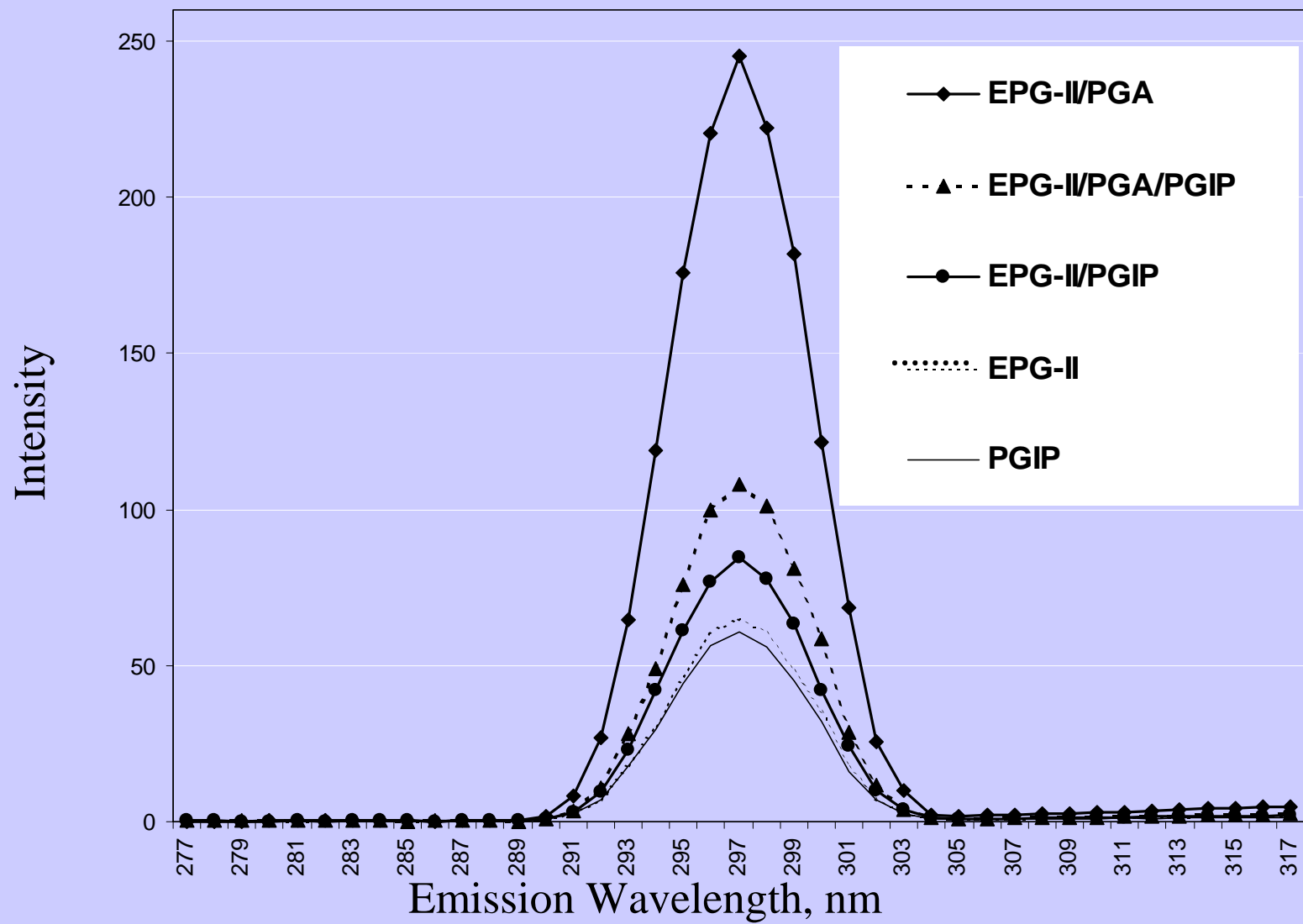
- ▶ The crystal structure of EPG-II from *A. niger* from the (a) top and (b) bottom.
 - Green: Amino acids in the binding cleft that were protected from exchange by substrate binding.
 - Gold: Amino acids that were exposed to exchange due to substrate binding
 - Red: The four residues that were protected from pepsin hydrolysis by PGIP binding



- Protected amino acids
- Protected cleavage sites
- Exposed amino acids
- Lysine Residues

Fluorescence

- ▶ Fluorescence spectra of various mutant EPG-II and PGIP complexes. The tryptophan emission spectra were excited at 290 nm.
- ▶ EPG-II/PGA signal is different from EPG-II/PGIP/PGA confirming the formation of a 3-component



Conclusions

- ▶ The underside of EPG-II is flexing upon substrate binding
- ▶ PGIP is able to restrain the conformational changes of EPG-II
- ▶ PGIP blocks cleavage sites on EPG-II to indicate the location of their interaction
- ▶ Results imply that PGIP acts as a non-competitive inhibitor

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