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Hot-spot labeling and mutations to gain insight into the protein dynamics of human blood group glycosyltransferases

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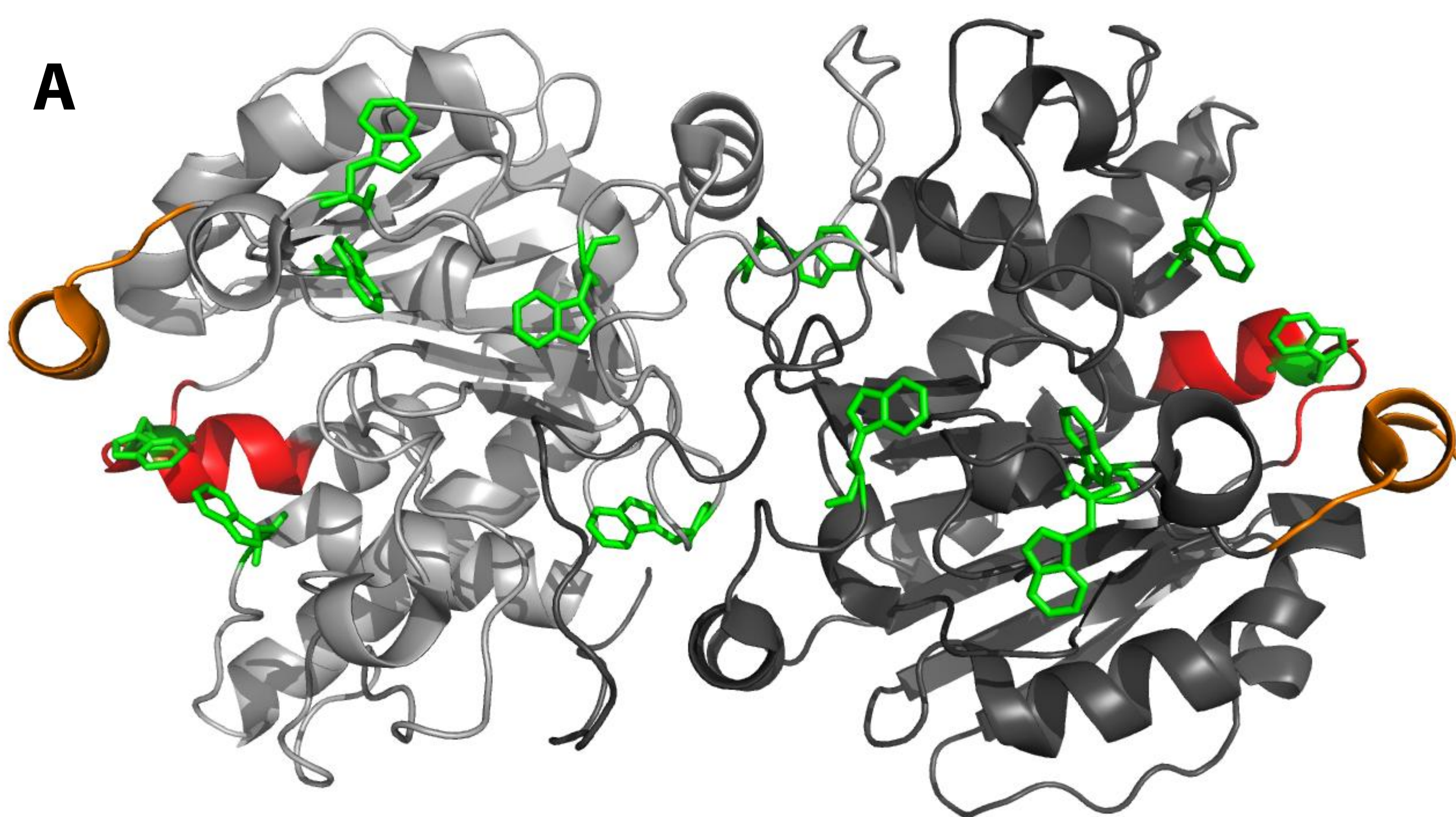
Introduction

Human α-(1→3)-*N*-acetylgalactosaminyltransferase (GTA) and α-(1→3)-galactosyl-transferase (GTB) catalyze the last step in enzymatic synthesis of the ABO(H) blood group A and B antigens, and although they use different donor substrates they differ only by four amino acid residues. GTA and GTB are retaining enzymes, and many details of their reaction mechanism remain to be elucidated.

High resolution X-ray crystallographic structures[1] show that these enzymes undergo significant molecular movements during catalysis and imply that understanding of molecular dynamics is a key to understand catalysis (Figure 1 B).

A powerful tool to monitor molecular dynamics is NMR spectroscopy. To gain information about molecular movements we introduced specifically labeled amino acids (Ile, Leu, Met) as probes at highly flexible or otherwise interesting positions ("hot spots").

Tryptophan is one of the most common amino acids in protein hot spots and involved in protein-ligand and protein-protein interactions. In this study tryptophan is chosen as probe because it is positioned on the tip of the highly flexible, so called "internal loop"(W181), and in the active site directly interacting with the acceptor substrate (W300, figure 1). The selective labeling can either be achieved by adding the amino acid or a metabolic precursor with the suitable isotope label to the medium.



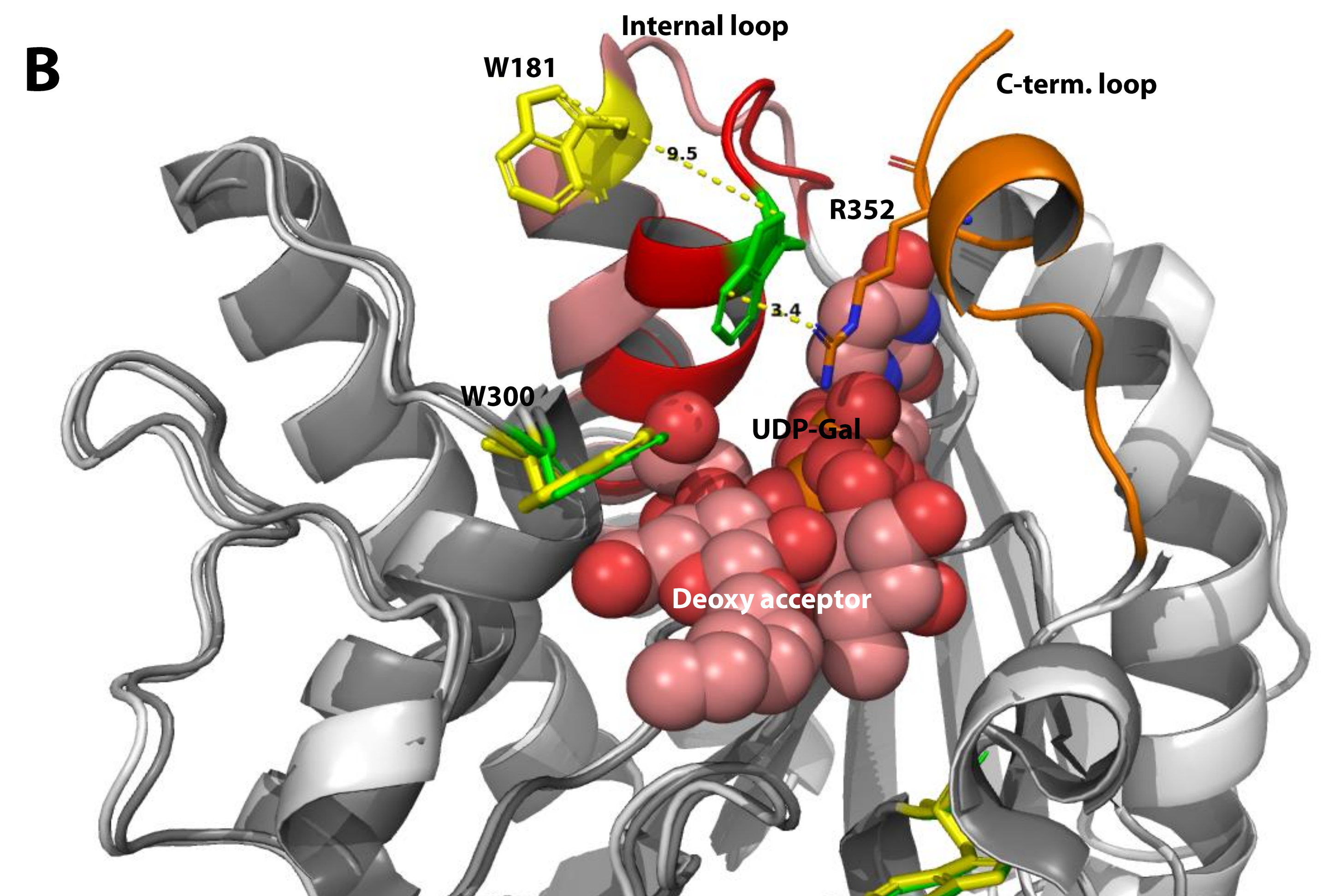


Figure 1: A) The soluble part of GTA/GTB forms very stabile homodimers with a MW of ca. 70kDa. Each subunit contains six tryptophan residues (green). **B)** Overlay of the crystal structures of the open (light grey, rose, yellow) and closed (dark grey, red, green) conformation of an GTA/GTB hybrid with ligands**.** The internal loop (red) and the C-terminal loop (orange) undergo significant molecular movements during substrate binding. To monitor this dynamics Trp181 is ideally suited, since it is positioned at the tip of the internal loop forming an cation-π-interaction with Arg352 in the closed conformation that restricts flexibility of the C-terminal loop. The donor substrate and the deoxy acceptor analog are displayed as spheres. (PDB IDs: 2RJ7 closed conformation, 3I0H open conformation)

Methods & Results

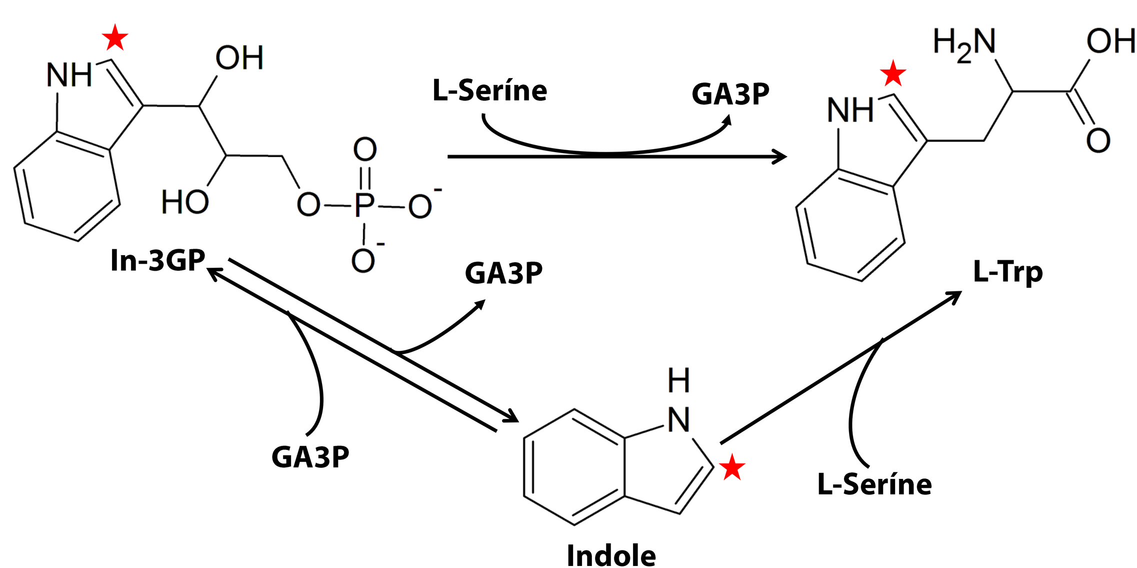


Figure 2: Trp sidechain labeling. *E.coli* tryptophan synthase catalyzes the last two steps in biosynthesis of tryptophan in a substrate channeling mode. Supplying the cells with labeled indole in the medium leads to incorporation of this precursor into tryptophan side chains. [2]

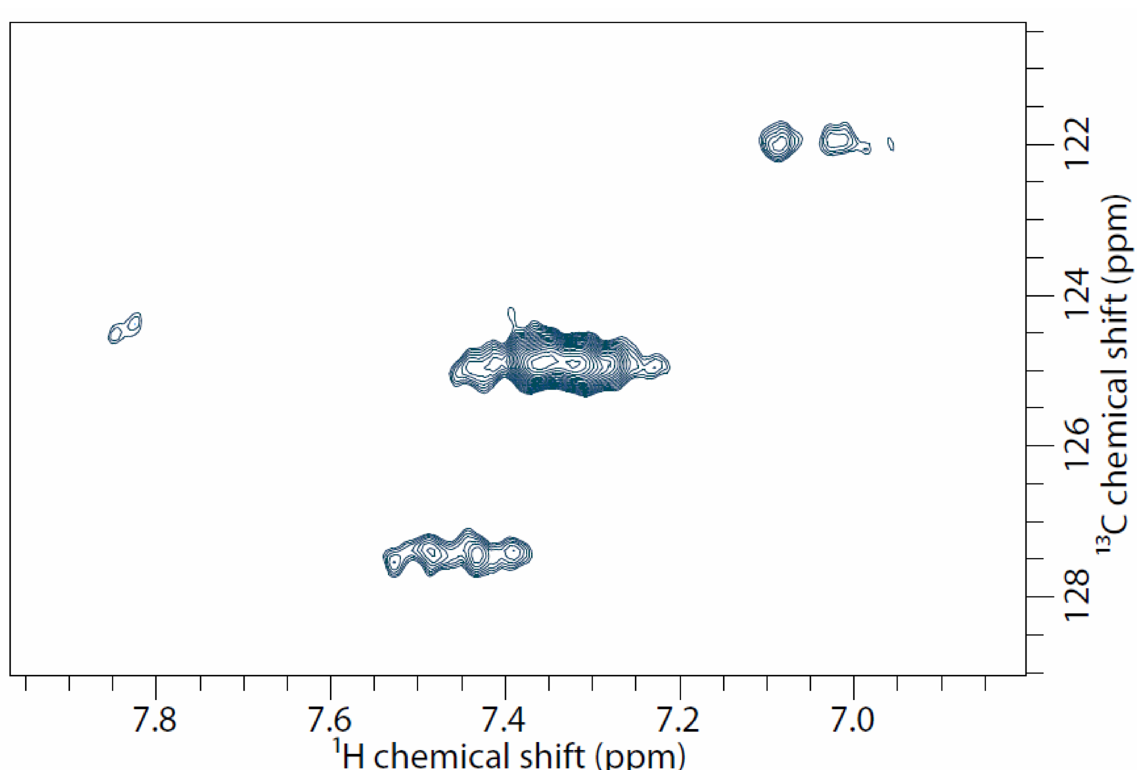
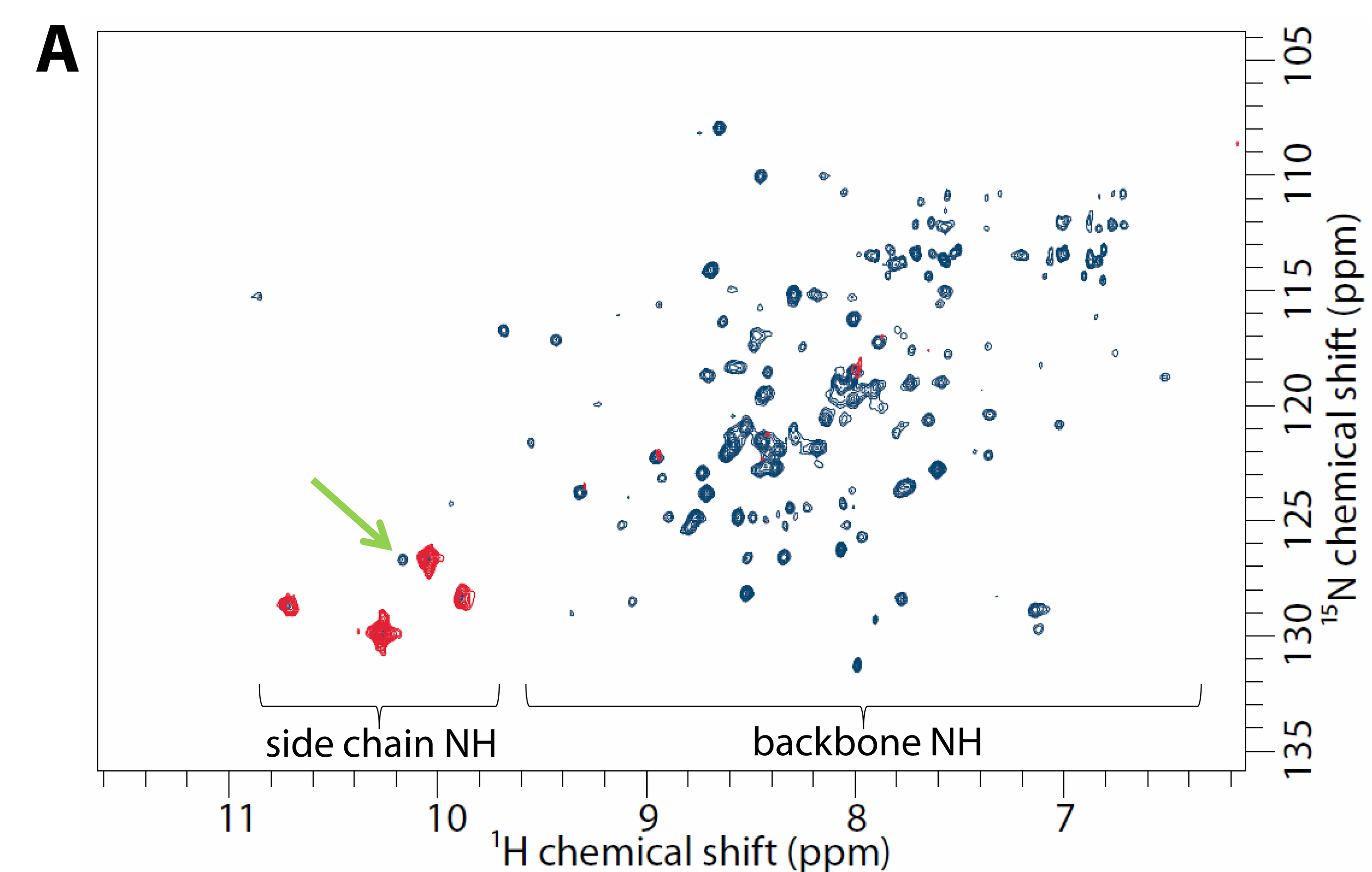
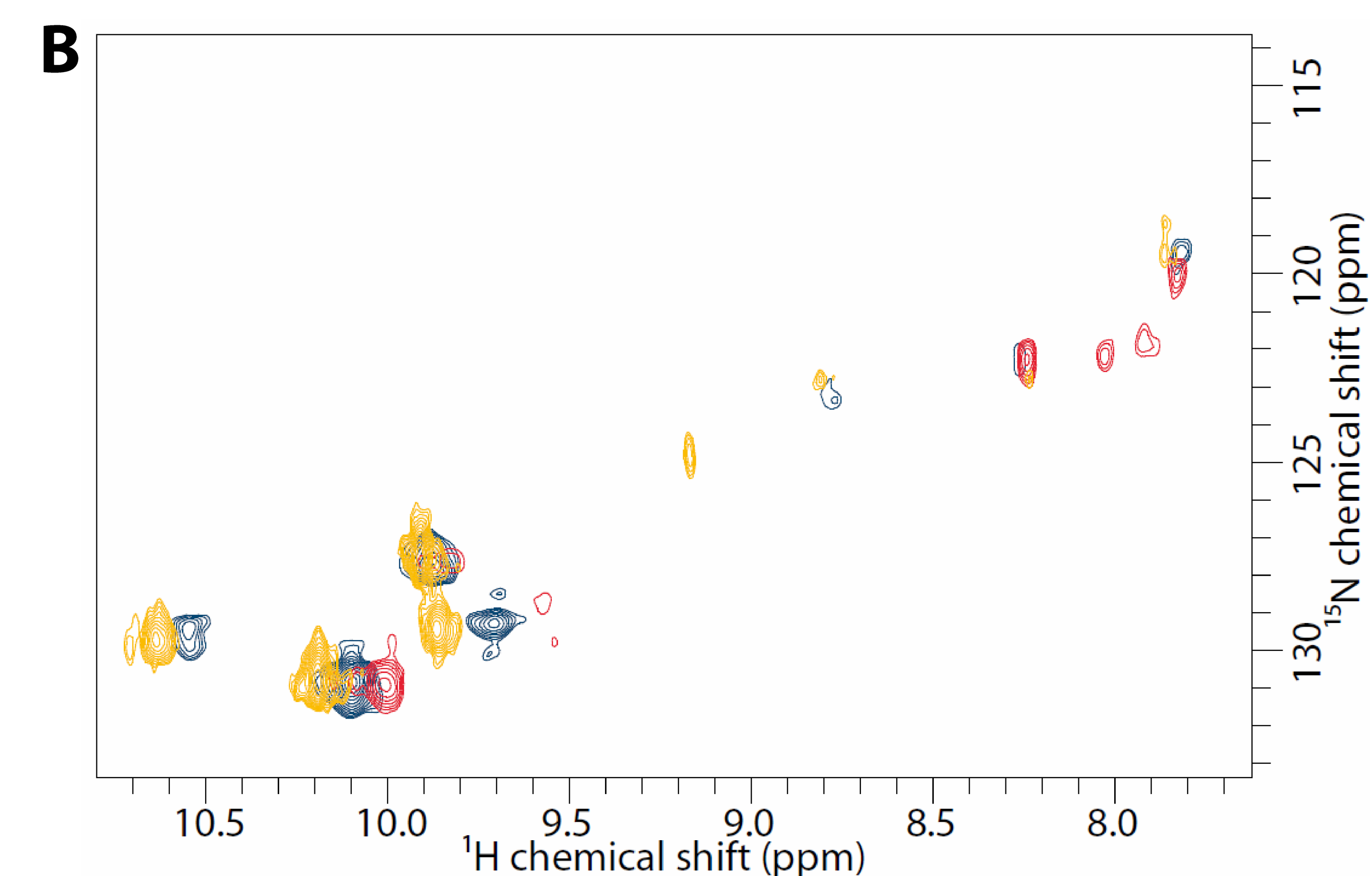


Figure 3: 2D [^{13}C,^{1}H]-HMQC recorded for uniformly deuterated, [^{1}H,^{13}C$_{\delta1}$]-tryptophan labeled ,^{15}N-GTB. Spectra were recorded with 0.7 mM GTB in 25 mM sodium phosphate 10% D_2O buffer 5 mM $MgCl_2$, 1mM β-ME-d_6, 100 μM DSS-d_4 at pH 6.7 and T = 298 K. The signals are quite broad on the ^{1}H chemical axis. Several conformational species could be responsible for this.

Figure 4: A) Overlay of 2D [^{1}H,^{15}N]-TROSY-HSQC spectra recorded for uniformly deuterated, [^{1}H,^{15}N]-tryptophan labeled GTB (red) and uniformly deuterated and ^{15}N-enriched GTB (blue). Protein NMR data suggest that Trp side chains show up at downfield chemical shifts. Peaks originating from Trp side chain NH-groups are readily identifiable. One peak in this area is not a Trp peak (green arrow), which could be confirmed by unlabeling (data not shown). Also backbone NH-groups could be detected but with a much less sensitivity. This is due to Trp degradation. These signals will help for assignment of the backbone.
Spectra were recorded with 0.5 mM GTB in 25 mM sodium phosphate 10% D_2O buffer 5 mM $MgCl_2$, 1mM β-ME-d_6, 100 μM DSS-d_4 at pH 6.7 and T = 298 K.

B) Overlay of 2D [^{1}H,^{15}N]-TROSY-HSQC spectra recorded for uniformly deuterated, [^{1}H,^{15}N]-tryptophan labeled GTB at T = 283 K (red), 298 K (blue) and 313 K (yellow). The intensity and the apparent chemical shift of the peaks is temperature dependent. The peaks can be divided into backbone and side chain signals. While the intensity of the side chain NHs increases with rising temperature, the intensity of backbone NHs decreases. This can be explained by a faster exchange between two conformations at higher temperatures which leads to a sharp peak. Also proton exchange is accelerated at higher temperatures which leads to decreasing signal intensity for backbone NHs.
Spectra were recorded with 0.5 mM GTB in 25 mM sodium phosphate 10% D_2O buffer 5 mM $MgCl_2$, 1mM β-ME-d_6, 100 μM DSS-d_4 at pH 6.7.





Conclusion & Outlook

First results are very promising and show that tryptophan labeling has a huge potential. Compared to ^{13}C labeling, ^{15}N labeling yields heteronuclear correlation spectra of much better sensitivity. For analysis of molecular dynamics it will be necessary to assign the peaks in the spectra. Titration NMR experiments with known ligands will be very helpful in the assignment process as well as site-directed mutagenesis. Of great interest will be a Trp181Met mutant because the cation-π-interaction, which stabilizes the two flexible loops in the closed conformation may no longer be formed. Opening and closure movements of the two flexible loops are at least partially expected to take place on the microsecond to millisecond time scale. Therefore, relaxation dispersion NMR experiments are well suited to study these motions.

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[2] Rodriguez-Mias, R. A. and M. Pellecchia, *Journal of the American Chemical Society* 125(10): 2892-2893 (2003).