

# Kinetics of the Antibody Recognition Site in the Third IgG-Binding Domain of Protein G

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Dedicated to Jacob Anglister on the occasion of his 65th birthday

**Abstract:** Protein dynamics occurring on a wide range of timescales play a crucial role in governing protein function. Particularly, motions between the globular rotational correlation time ( $\tau_c$ ) and 40  $\mu$ s (supra- $\tau_c$  window), strongly influence molecular recognition. This supra- $\tau_c$  window was previously hidden, owing to a lack of experimental methods. Recently, we have developed a high-power relaxation dispersion (RD) experiment for measuring kinetics as fast as 4  $\mu$ s. For the first time, this method, performed under super-cooled conditions, enabled us to detect a global motion in the first  $\beta$ -turn of the third IgG-binding domain of protein G (GB3), which was extrapolated to  $371 \pm 115$  ns at 310 K. Furthermore, the same residues show the plasticity in the model-free residual dipolar coupling (RDC) order parameters and in an ensemble encoding the supra- $\tau_c$  dynamics. This  $\beta$ -turn is involved in antibody binding, exhibiting the potential link of the observed supra- $\tau_c$  motion with molecular recognition.

Nature has very likely exploited the intrinsic flexibility of biomolecules, which sample various structural states over a wide-range of timescales (spanning from picoseconds to hours)<sup>[1–3]</sup> for the purpose of functionality, that is, enzymatic

catalysis<sup>[4–6]</sup> and molecular recognition.<sup>[7,8]</sup> In regard to molecular recognition, experimental evidence is accumulating that suggests an important role for conformational sampling for the purpose of productive binding events, specifically within the supra- $\tau_c$  window (between the molecular tumbling time ( $\tau_c$ ) and ca. 40  $\mu$ s).<sup>[7,9,10]</sup> Recent work on ubiquitin has demonstrated how the entire protein behaves as an allosteric switch geared toward binding partner selection, by regulating constructive molecular recognition through the expansion and contraction of the protein.<sup>[11]</sup> NMR spectroscopy has been an invaluable tool for providing the experimental observations necessary for connecting structural dynamics with molecular recognition, through relaxation dispersion (RD) and residual dipolar coupling (RDC) measurements.

Herein, we investigate the linkage between supra- $\tau_c$  motion and molecular recognition for the third immunoglobulin (IgG) binding domain of protein G (GB3). As one of three IgG binding domains, GB3 plays an important role in antibody recognition<sup>[12]</sup> with applications in immunoprecipitation.<sup>[13]</sup> Earlier studies on GB3 have indicated the presence of elevated dynamics within the supra- $\tau_c$  window, particularly for a stretch of residues (G9-K13) connecting  $\beta$ -strands 1 and 2, but until now the kinetics could not be measured.<sup>[14–19]</sup> Here, a series of high-power RD experiments performed at super-cooled temperatures allow us to determine the kinetics of conformational exchange within the GB3 ground state for residues G9-K13. Next, we utilized previously measured RDCs to generate an ensemble-restrained molecular dynamics (ERMD) ensemble of GB3, allowing us to interpret the nature of the measured supra- $\tau_c$  motion. Finally, we relate the experimentally observed supra- $\tau_c$  conformational dynamics of GB3 to molecular recognition of an antibody.

Although RD measurements are routinely employed to study the relationship between bio-molecular conformational inter-conversion and functionality, the technique could only probe motions slower than about 40  $\mu$ s owing to limitations on the maximum achievable radio frequency (RF) field.<sup>[20]</sup> Recently, we have shown that cryogenically cooled probeheads can safely handle even higher RF fields<sup>[21]</sup> enabling the observation of kinetic processes occurring on timescales an order of magnitude faster (ca. 4  $\mu$ s).<sup>[22]</sup> The aforementioned technological advancements in RD are quite timely given the current focus on linking the process of molecular recognition with conformational sampling within the ground state ensemble of a protein, particularly motions occurring between the

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molecular tumbling time ( $\tau_c$ ) and the temporal limit of RD (termed the supra- $\tau_c$  window). This method can be further enhanced by two augmentations to facilitate the RD measurement, either via increasing the chemical shift variance within the probed ensemble (with a paramagnetic tag<sup>[23]</sup> or through increasing the  $B_0$  field)<sup>[24]</sup> or lowering the temperature to slow down the kinetic process (super-cooled conditions).<sup>[25]</sup> In the following, for the first time we have combined high-power RD measurements with super-cooled temperatures to detect a kinetic process in GB3 that would otherwise be beyond the limits for observation.

High-power RD experiments on GB3 backbone amide  $^{15}\text{N}$  resulted in flat dispersion profiles for all observed residues at 275 K (data not shown), suggesting either the absence of conformational exchange in GB3 within the detectable timescale window ( $> 25\ \mu\text{s}$ ) or the chemical shift variance (CSV) for the backbone amide  $^{15}\text{N}$  within the GB3 ensemble is too small for detecting RD. From here, we performed the high-power RD measurements on backbone amide  $^1\text{H}$ , enabling us to extend the detectable time-scale window by an order of magnitude ( $> 3.7\ \mu\text{s}$  when applying a maximum effective radiofrequency field of  $272\,000\ \text{rad s}^{-1}$ ). At 275 K,<sup>[22]</sup> significant conformational exchange in GB3 was detected for residues K9–G13 (gray lines in Figures 1A–E) spanning the first  $\beta$ -turn region (Figure 1G), whereas all other residues produced flat dispersion curves. The five RD profiles were fitted to a single  $\tau_{\text{ex}}$  of  $9.1 \pm 0.4\ \mu\text{s}$  following an Akaike information criterion ( $\text{AIC}_C$ ) based clustering approach (in house).

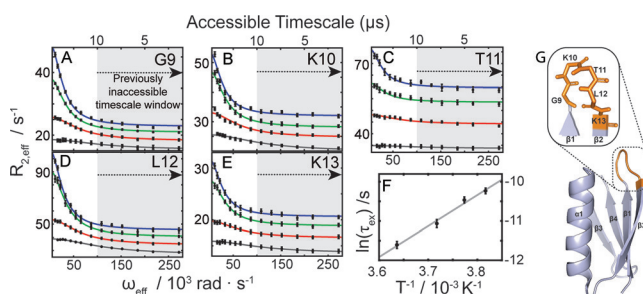
To investigate the temperature dependence of  $\tau_{\text{ex}}$ , we measured high power RD with GB3 under super-cooled conditions at three additional temperatures: 262, 265, and

269 K, since the experimentally measured timescale of motion at 275 K ( $9.1 \pm 0.4\ \mu\text{s}$ ) is almost at the edge of the detectable limit ( $3.4\ \mu\text{s}$ ) barring the possibility of performing experiments at higher temperatures. At the three additional temperatures, only the same stretch of five residues (K9–G13) displayed detectable RD (Figure 1A–E). As with the RD data at 275 K, the RD profiles of all five  $\beta$ -turn residues at each temperature could be fitted to a single  $\tau_{\text{ex}}$  of:  $35.9 \pm 1.3$ ,  $28.5 \pm 1.1$ , and  $15.6 \pm 0.8\ \mu\text{s}$  at 262, 265, and 269 K, respectively. In all cases, a single amino acid fit or a global fit gave similar results (Supporting Information, Tables S1, S2).

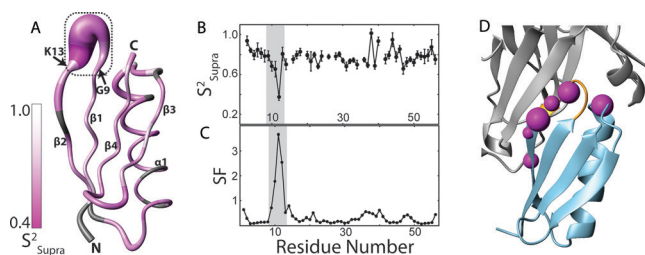
With the temperature-dependent global  $\tau_{\text{ex}}$  for these  $\beta$ -turn residues, an Arrhenius-type equation was used to estimate the activation energy. It should be noted that the kinetic process we are measuring with the high-power RD involves a continuum of states that we assume all interconvert with the same activation energies and attempt frequencies. Utilizing either the Arrhenius equation or the Eyring equation (see Supporting Information and Figure S1) will only precisely provide the activation energy or the Gibbs free energy, respectively. For GB3, we estimate the activation energy for conformational exchange as  $65.6 \pm 4.7\ \text{kJ mol}^{-1}$  and extrapolated  $\tau_{\text{ex}}$  to physiological temperature (310 K) as  $371 \pm 115\ \text{ns}$ .

A key complement to the RD measurements are RDCs, which report on the amplitude of inter-nuclear vector motion relative to the external magnetic field within the ps–ms timescale.<sup>[2]</sup> RDCs can be used as a probe to characterize supra- $\tau_c$  protein dynamics, both in a model-free manner<sup>[15]</sup> and as restraints in the generation of conformational ensembles;<sup>[7,26]</sup> however, RDCs lack the exact temporal information provided by the RD measurements. Elevated supra- $\tau_c$  dynamics for GB3 encoded by RDCs can be encapsulated by an order parameter quantifying the amount of supra- $\tau_c$  motion present in each  $\text{H}^{\text{N}}\text{--N}$  bond vector, termed  $S_{\text{Supra}}^2$ , which is calculated by normalizing the previously reported order parameters  $S_{\text{RDC}}^2$ <sup>[15]</sup> (amplitude of vector motion within the ps to ms timescale) with  $S_{\text{LS}}^2$ <sup>[27]</sup> (amplitude of vector motion within the ps to  $\tau_c$  timescale) ( $S_{\text{Supra}}^2 = S_{\text{RDC}}^2/S_{\text{LS}}^2$ ). As shown by Figure 2A and 2B, almost every  $\text{H}^{\text{N}}\text{--N}$  bond vector in GB3 possesses a significant amount of motion slower than the rotational correlation time, particularly in loop region connecting  $\beta$ -strands 1 and 2.

To represent the structural heterogeneity of GB3 indicated by  $S_{\text{Supra}}^2$ , we calculated an ensemble-restrained molecular dynamics (ERMD) ensemble of GB3 in a similar manner as the ERNST ensemble of ubiquitin,<sup>[26]</sup> utilizing the same RDC data set<sup>[14]</sup> as with the model-free ORIUM<sup>[15]</sup> evaluation of GB3 (see the Supporting Information for details on the ensemble generation). For the purposes of cross-validation, we measured *trans*-hydrogen bond scalar couplings ( $^3\text{hJ}_{\text{NC}}$ ) for GB3, since all previous analyses of GB3 ensembles utilized  $^3\text{hJ}_{\text{NC}}$  taken from the first immunoglobulin (IgG) binding domain of protein G (GB1; Supporting Information, Table S3). Table S4 in the Supporting Information tabulates the level of agreement between ERMD and the reported NMR observables, demonstrating the high quality of the ensemble and justifying its use in depicting the nature of the motion within the first  $\beta$ -turn region.



**Figure 1.** Temperature dependence of the observed supra- $\tau_c$  dynamics in GB3. High-power  $R_{1\rho}$  relaxation dispersion (RD) experiments on backbone amide protons of GB3 revealing previously undetected dynamics within the first  $\beta$ -turn for residues G9 (A), K10 (B), T11 (C), L12 (D), and K13 (E). Shaded areas indicate the recently developed high-power  $R_{1\rho}$  RD experiment, which can be used to investigate motion within the previously inaccessible dynamics window. Solid lines represent fits of the experimental data utilizing a second-order Akaike's information criterion ( $\text{AIC}_C$ ), where all RD data at a single temperature were fitted to a single global exchange lifetime ( $\tau_{\text{ex}}$ ) (see the Supporting Information, Table S1 for fitted values). Experimental temperatures are denoted by the following colors: gray 275 K, red 269 K, green 265 K, blue 262 K. F) Plot of  $\tau_{\text{ex}}$  versus temperature. The solid gray line indicates the fit of the experimental data to the Arrhenius equation; the activation energy was calculated as  $65.6 \pm 4.7\ \text{kJ mol}^{-1}$ . G) The residues showing significant RD are depicted on a structure of GB3 (PDB ID: 2OED) in orange.



**Figure 2.** GB3 supra- $\tau_c$  dynamics and a potential link to molecular recognition. Residual dipolar coupling (RDC) derived structural ensemble of GB3 exhibits enhanced fluctuations in the first  $\beta$ -turn region that shows RD. A) The ERMD (ensemble-restrained molecular dynamics) ensemble, containing 640 structures, is shown as a tube (axis: mean position of the backbone C $\alpha$  atoms; radius: square fluctuation of the backbone C $\alpha$  atoms). The ensemble is generated from previously measured RDCs.<sup>[14]</sup> The tube is colored in magenta according to the amount of extra supra- $\tau_c$  mobility ( $S^2_{\text{Supra}}$ ) every residue has compared to the LS model.  $S^2_{\text{Supra}}$  is calculated by normalizing the RDC derived order parameter,  $S^2_{\text{RDC}}$ ,<sup>[15]</sup> with  $S^2_{\text{LS}}$ <sup>[27]</sup> ( $S^2_{\text{Supra}} = S^2_{\text{RDC}}/S^2_{\text{LS}}$ ) and plotted against residue number in (B). C) Principle component analysis (PCA) carried out on the ERMD ensemble and the square fluctuation (SF) along PC1. In both (B) and (C) the shaded area denotes the stretch of residues that shows RD at all temperatures from 262 K to 275 K. All of ERMD,  $S^2_{\text{Supra}}$ , and SF patterns independently verify that the first  $\beta$ -turn (boxed region) displays the most supra- $\tau_c$  mobility and fluctuation as measured by RD. D) GB3 (sky blue) in complex with a Fab fragment (gray) from a selected part of the crystal structure 1IGC. The stretch of residues showing relaxation dispersion is shown in orange. Magenta spheres with various radii are employed to indicate the number of contacts (within 5 Å) between the backbone amide protons of GB3 and the binding partner.

Previously, we have shown that ubiquitin RD data could be rationalized by CSVs within a RDC restrained conformational ensemble assuming, for simplicity, a single rate of interconversion between the ensemble members.<sup>[9]</sup> Furthermore, this strategy was even able to single out the most probable motion within ubiquitin responsible for the detected RD.<sup>[11]</sup> Thus, we calculated the CSV for both  $^1\text{H}$  and  $^{15}\text{N}$  from the 640 member ERMD ensemble using the chemical shift predictors SHIFTX<sup>[28]</sup> and SPARTA<sup>[29]</sup> (Supporting Information, Figure S2). Since the underlying dynamics are on a faster time-scale than the detection limit for  $^{15}\text{N}$  nuclei, no experimental CSVs were available. For the  $^1\text{H}$  nuclei of the loop for which RD was observable, the relative sizes of predicted and experimental CSVs match well except for L12 amide which has the highest experimental CSV but a small predicted CSV. The large experimental CSV value for the amide proton of L12 indicates the forming and breaking of a hydrogen bond with the carbonyl oxygen of G9. However, the ensemble calculated CSV did not show this process, which may be due to the inaccuracy of hydrogen-bonded proton chemical shift prediction.<sup>[30]</sup> It also should be noted that when removing L12 from calculation of the correlation between the SPARTA determined CSV versus the experimental CSV resulted in correlation coefficients ranging from 0.74 to 0.94 over the four temperatures (Supporting Information, Figure S3). For all other amides, no relaxation dispersion is observed which is the easiest explanation is to assume that the supra- $\tau_c$  dynamics is faster than our present limit of 4  $\mu\text{s}$ .

Principle component analysis (PCA) was performed on the ERMD ensemble (Supporting Information, Figure S4) and the square-fluctuation for each backbone C $\alpha$  atom was determined along the largest mode (PC1; Figures 2C). The main difference between a root-mean-square difference (RMSD) analysis and a principle component analysis (PCA) is that RMSD is an indication of local variability from a mean structure (Supporting Information, Figure S5), while PCA considers the entire protein ensemble to describe a global motion within a reduced dimensionality. In the case of GB3, the first principle component (PC1) illustrates the directionality of the motion captured by the ensemble restrained with RDCs that encode supra- $\tau_c$  dynamics and predicts two unique clusters that could be exchanging between each other, primarily through the first  $\beta$ -turn region, on the timescale we have measured with RD.

GB3 is located in the immunoglobulin (antibody) binding domain of Protein G, and residues in the first  $\beta$ -turn and in the following  $\beta$ -strand ( $\beta_2$ ) of GB3 are known to play important roles in antibody recognition.<sup>[12]</sup> The crystal structure of GB3 (light blue) in complex with an antibody (Gray, Fab fragment; MOPC21) is shown in Figure 2D (PDB ID: 1IGC).<sup>[12]</sup> We calculated the number of contacts within 5 Å per amide proton of GB3 with its binding partner (indicated by the radius of the red spheres) and plotted them for each residue (Supporting Information, Figure S6). The contact map shows that GB3 recognizes the Fab fragment mainly through two binding sites: the first  $\beta$ -turn region and the C-terminal region of the  $\alpha$ -helix. Curiously, the amide protons in the  $\beta_2$  strand do not show detectable RD, despite the specified contacts with the Fab fragment and the predicted CSV (Supporting Information, Figure S2). There are two possible explanations, namely that either the actual CSV is not large enough to detect RD or there is a much faster process underlying the motion for this region. Regardless, what has been detected by RD is supra- $\tau_c$  motion within the first  $\beta$ -turn region from the antibody recognition site of GB3 that was indicated by  $S^2_{\text{Supra}}$  and was characterized by ERMD.

It is becoming evident that the linkage between sub- $\tau_c$  motion and functionality is entropic in nature,<sup>[8,31]</sup> whereas, supra- $\tau_c$  dynamics define the time-scale of conformational exchange between functional and non-functional states<sup>[4,11]</sup> and modulate the distribution of sub- $\tau_c$  conformational entropy.<sup>[22,32]</sup> Within this context, our results suggest a connection between GB3 conformational inter-conversion within the ground state ensemble ( $371 \pm 115$  ns at 310 K, extrapolated with the Arrhenius equation) and antibody recognition. Through the first demonstration of measuring high power RD at super-cooled temperatures, we were able to observe ground state kinetic transitions, in this case hundreds of nanoseconds at physiological temperatures, by extrapolation from low temperatures. By combining ensemble representations of proteins encompassing supra- $\tau_c$  dynamics with high power RD measurements, we anticipate that this method will be applicable for a wide-range of systems, which are currently lacking characterization of motions within this important timescale window, as long as the system is tractable for NMR and is amenable to perdeuteration.

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