

# Aromatic ring flips reveal reshaping of protein dynamics in crystals and complexes

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Protein conformational energy landscapes are shaped not only by intramolecular interactions but also by their environment. In protein crystals and protein–protein complexes, intermolecular contacts alter this energy landscape, but the exact nature of this alteration is difficult to decipher. Understanding how the crystal lattice affects protein dynamics is crucial for crystallography-based studies of motion, yet its influence on collective motions remains unclear. Aromatic ring flips in the hydrophobic core represent sensitive probes of such dynamics. Here, we compare the kinetics of aromatic ring flips in the protein GB1 in crystals, in complex with its binding partner IgG, and in solution, combining advanced isotope labelling with quantitative NMR methods. We show that rings in the core flip nearly a thousand times less frequently in crystals than in solution. Enhanced-sampling molecular dynamics simulations, based on a crystal structure of a GB1 variant reported in this work, reproduce these elevated barriers and reveal how the crystal restrains motions.

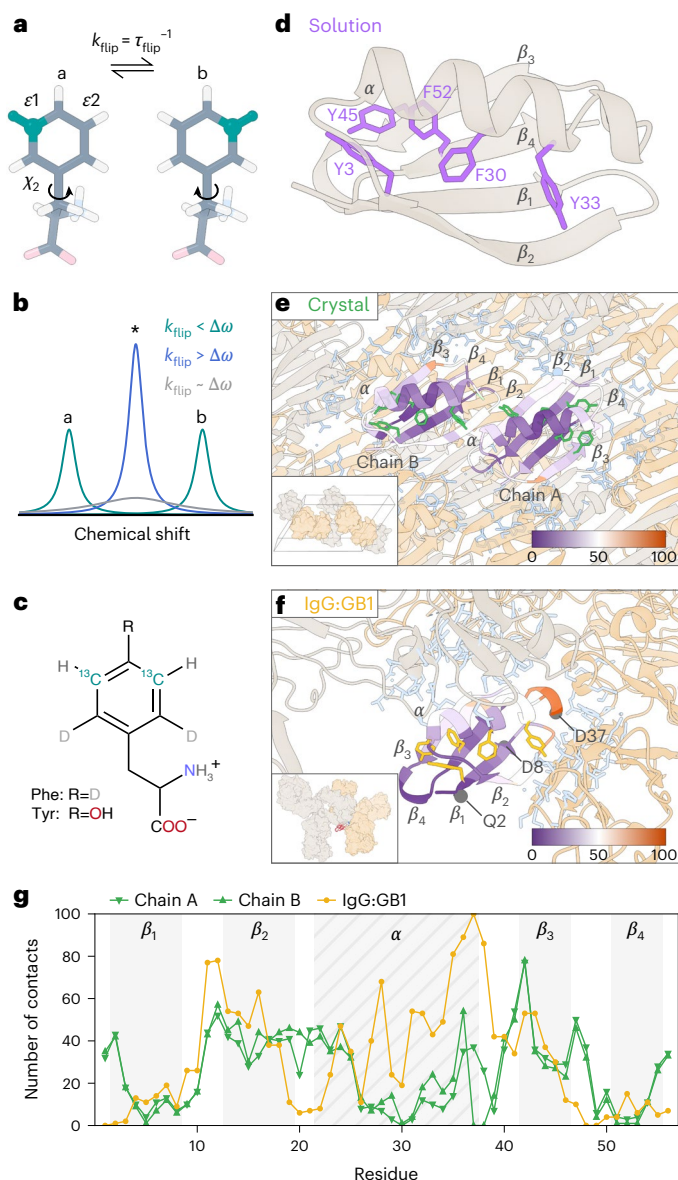
Protein crystallography has been the central pillar of structural biology for over half a century, with two hundred thousand structures determined up to now<sup>1</sup>. It has been recognized early on that these seemingly static images are misleading; as G. Weber expressed in 1975, proteins in solution are ‘kicking and screaming stochastic molecules’<sup>2</sup>. Meanwhile, proteins in crystal lattices still exhibit substantial motion, which can be indirectly observed via residue-wise B-factor variability, blurring of the electron density or co-existing conformations (so-called alternate locations)<sup>3–5</sup>. A long-standing question is to what extent crystal packing constrains protein dynamics relative to solution<sup>6,7</sup>. Early studies of enzymatic activity<sup>8–14</sup> revealed a close match between crystal and solution in some cases<sup>15</sup>, and strongly attenuated activity in others<sup>8</sup>. Comparisons of different crystal forms established that packing can alter both structure and dynamics<sup>16–18</sup>. Today, serial crystallography at X-ray free-electron lasers and synchrotrons enables the capture

of protein kinetics and mechanisms in crystals at room temperature with up to femtosecond time resolution<sup>19–30</sup>. These experiments build upon the assumption that the crystal lattice does not influence protein dynamics and underline the relevance of this question once again. Crystallographic methods can naturally not assess the dynamics of non-crystalline samples, and therefore cannot quantify constraints of the lattice on protein motions relative to solution.

Molecular dynamics (MD) simulations of entire crystal unit cells show that proteins retain substantial internal flexibility<sup>31–37</sup>. However, MD currently accesses only motions up to the  $\mu$ s regime, leaving slower conformational changes, often central to function, difficult to capture.

Nuclear magnetic resonance (NMR) spectroscopy can probe protein dynamics in various environments. Comparisons of solution-state NMR with magic-angle spinning (MAS) NMR of protein crystals yield direct insights into the modulation of motions by the crystal lattice.

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**Fig. 1 | Specific isotope labelling of aromatic residues in different forms of GB1.** **a**, Schematic representation of a ring flip. The  $\chi_2$  angle around the  $C^\beta$ – $C'$  bond rotates  $180^\circ$  with a flip rate  $k_{\text{flip}}$ .  $(\text{CH})^{\epsilon 1}$  and  $(\text{CH})^{\epsilon 2}$  exchange their position in the ring, creating states **a** and **b**. **b**, Schematic timescale-dependent NMR signatures resulting from flips of  $(\text{CH})^\epsilon$ -labelled Phe or Tyr residues. Signals of sites **a** and **b** are resolved if  $k_{\text{flip}}$  is slower than the chemical shift difference  $\Delta\omega$  (cyan). If  $k_{\text{flip}} > \Delta\omega$ , the signals are averaged (blue). At intermediate timescales ( $k_{\text{flip}} \sim \Delta\omega$ ), the signal is broadened (grey). **c**,  $\alpha$ -Ketoacid precursor for  $3,5\text{-}^{13}\text{CH}$  labelling of the aromatic ring of Phe and Tyr<sup>47</sup>. **d**, Structure of  $\text{GB1}_{\text{QDD}}$  in solution with Phe and Tyr as purple sticks. Secondary structure elements are indicated in grey. **e**, Surrounding of  $\text{GB1}_{\text{QDD}}$  (asymmetric unit with chains A and B) in the crystal lattice. The backbone colour represents the graph in **g**. Atoms within  $10 \text{ \AA}$  of  $C^\alpha$  atoms are shown in light blue sticks. The insert shows one unit cell with A and B molecules in different colours. **f**, Surrounding of  $\text{GB1}_{\text{QDD}}$  in IgG:GB1 (backbone colour as explained above).  $C^\alpha$  atoms of Q2, D8 and D37 are shown as grey spheres. The insert shows an overview of the complex (modelled as described in ref. 53). **g**, Number of atoms within  $10 \text{ \AA}$  of  $C^\alpha$  atoms in chains A and B of the crystal and the IgG:GB1 complex. Secondary structure elements are indicated as in the structure representations in **d–f**.

Spin-relaxation experiments showed that fast (sub- $\mu\text{s}$ ) local motions are largely preserved in crystals, except in loops engaged in packing contacts<sup>18,38,39</sup>. Slower motions on the  $\mu\text{s}$ –ms timescale seem to be

more strongly perturbed: the  $\beta$ -turn in ubiquitin is slowed by more than an order of magnitude in crystals, and the populations are space group dependent<sup>40,41</sup>.

Aromatic rings of phenylalanines and tyrosines undergo flips around the  $C^\beta$ – $C'$  axis (the  $\chi_2$  angle; Fig. 1a)<sup>42–44</sup> and are particularly sensitive probes of collective dynamics. Buried rings are thought to require large, cooperative motions of the protein to flip, called ‘breathing motions’<sup>45,46</sup>. Because the two interchanging states are structurally indistinguishable, crystallography cannot detect these flips. By contrast, they are easily observable by NMR, since the  $^1\text{H}$  and  $^{13}\text{C}$  spins on each side of the aromatic ring (for example,  $\epsilon_1$  and  $\epsilon_2$ ) differ in chemical shift. A ring flip consequently leads to an interchange of  $(\text{CH})^{\epsilon 1}$  and  $(\text{CH})^{\epsilon 2}$  as well as  $(\text{CH})^{\delta 1}$  and  $(\text{CH})^{\delta 2}$ , resulting in timescale-dependent spectral signatures (Fig. 1b). The introduction of  $\alpha$ -ketoacid precursors (Fig. 1c) for site-specific labelling of the  $(\text{CH})^\epsilon$  atoms has greatly enhanced the precision of such studies<sup>47</sup>.

Here we study aromatic ring flips to assess how protein–protein interactions modify dynamics. We focus on the immunoglobulin-binding domain of protein G (GB1), studied in three contexts: in solution (Fig. 1d), the crystalline state (Fig. 1e), and in complex with its natural binding partner, the immunoglobulin G (IgG; Fig. 1f). Y3, F30, Y45 and F52 form a hydrophobic aromatic cluster, while Y33 is exposed to the solvent (Fig. 1d). The commonly studied T2Q variant ( $\text{GB1}_{\text{T2Q}}$ ) has been extensively characterized structurally and by solution and MAS NMR.  $\text{GB1}_{\text{QDD}}$ , a stabilized triple mutant (T2Q, N8D and N37D)<sup>48</sup>, is structurally uncharacterized, but temperature- and pressure-dependent solution NMR results provide a detailed baseline for comparison<sup>49,50</sup>.

Our integrated approach, combining solution and MAS NMR, crystallography and multi- $\mu\text{s}$  MD simulations, shows that crystal packing slows ring flips of buried residues by approximately three orders of magnitude. A thermodynamic and structural analysis of this observation provides insights into the origins of the elevated free-energy barrier in crystals. Remarkably, in the IgG complex, the same ring flips are much faster than in crystals, highlighting how the precise nature of intermolecular contacts reshapes the underlying free-energy landscape.

## Results

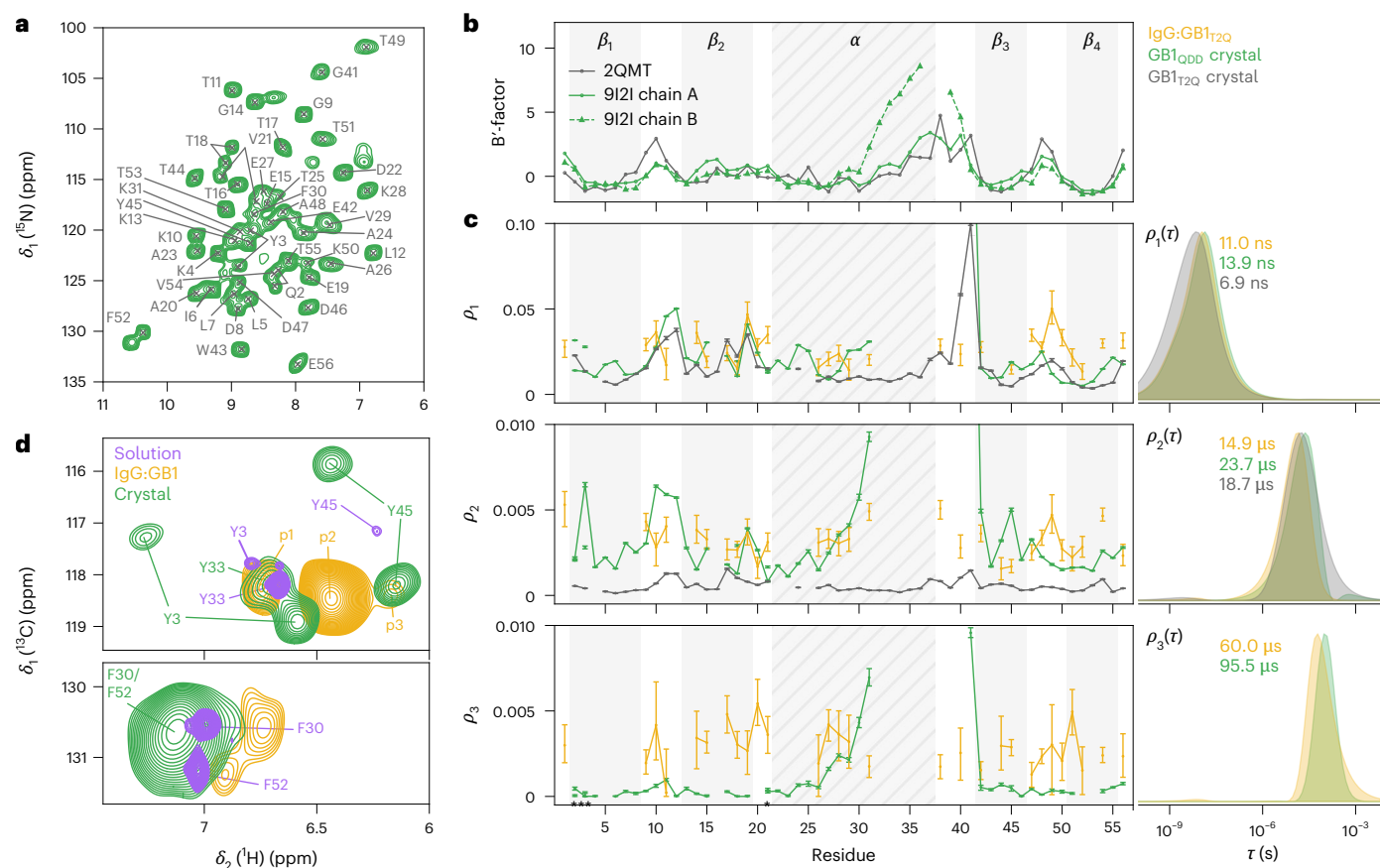
### The packing of $\text{GB1}_{\text{QDD}}$ in crystals and the IgG:GB1<sub>QDD</sub> complex

Precise atomic-resolution structures are important for the mechanistic interpretation of protein dynamics. To our knowledge, crystallization of  $\text{GB1}_{\text{QDD}}$  has not been previously reported. The  $\text{GB1}_{\text{T2Q}}$  variant yields crystals in the  $P3_121$  space group, suitable for solid-state NMR studies<sup>51</sup>. Crystallization of  $\text{GB1}_{\text{QDD}}$  reproducibly failed under these conditions, which we attribute to a disruption of packing interactions by the additional surface-exposed N8D and N37D mutations. Instead, we obtained  $\text{GB1}_{\text{QDD}}$  crystals in the  $C121$  space group using a dialysis-based method<sup>52</sup>. These crystals were suitable for MAS NMR and single-crystal X-ray diffraction (XRD), depending on the duration of crystallization (Supplementary Figs. 1 and 2; see Methods for details). Single-crystal XRD data were collected at a resolution of  $1.08 \text{ \AA}$  at  $100 \text{ K}$ , and  $1.69 \text{ \AA}$  at  $295 \text{ K}$  (Supplementary Table 1), revealing an asymmetric unit with two molecules (Fig. 1e).

A structural comparison between the crystal and complex revealed differences in intermolecular packing. We determined the number of non-hydrogen atoms within  $10 \text{ \AA}$  of each  $C^\alpha$  atom for chains A and B in the crystal (Fig. 1e) and in a model of the IgG:GB1 complex<sup>53–55</sup> (Fig. 1f). The two crystallographic chains exhibit very similar packing densities (Fig. 1g, green), but the distribution of contacts differs from that in the complex (yellow). These differences in the local packing could potentially modulate protein dynamics.

### MAS NMR of $\text{GB1}_{\text{QDD}}$ crystals

Equipped with this structural knowledge, we prepared crystals with different isotope-labelling schemes. For studies of aromatic residues,



**Fig. 2 | MAS NMR of different forms of GB1.** **a**, Dipolar-based  $^1\text{H}$ - $^{15}\text{N}$  NMR spectra of crystalline GB1<sub>QDD</sub> with assignment. **b**, Normalized B-factors of the GB1<sub>T2Q</sub> (grey, PDB 2QMT) and the GB1<sub>QDD</sub> (green, PDB 9I2I) crystal structures (normalization with BANAIT online tool<sup>82</sup>; see Supplementary Fig. 2 for raw B-factors). **c**, Detectors analysis of backbone-amide  $^{15}\text{N}$  spin-relaxation data for the IgG:GB1<sub>T2Q</sub> complex (yellow, 300 K)<sup>57,59,60</sup>, GB1<sub>QDD</sub> microcrystals (green, 304 K) and GB1<sub>T2Q</sub> microcrystals (grey, 300 K)<sup>57,58</sup>. The panels on the left show residue-

wise responses to the corresponding detector sensitivities  $\rho_{1-3}(\tau)$  on the right.  $\rho_{1-3}(\tau)$  differ slightly for each sample state, as the recorded relaxation rates were different. The error bars of the responses represent the 5–95% interpercentile range for Monte Carlo simulations with 200 iterations. **d**, Tyrosine (upper panel) and phenylalanine (lower panel) region of  $^1\text{H}$ - $^{13}\text{C}$  solution-state or dipolar-based MAS NMR spectra of  $(^{13}\text{C})$ -labelled GB1 in solution (purple), a complex with IgG (yellow) and a microcrystal (green).

we used metabolic precursors to obtain isolated  $(^{13}\text{C})$ -spin pairs in phenylalanines and tyrosines in addition to uniform  $^2\text{H}$  and  $^{15}\text{N}$  labelling (Fig. 1c; see Methods for details)<sup>47</sup>. The protein used for backbone assignment was labelled uniformly with  $^2\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$  (exchangeable hydrogens were  $^1\text{H}$ ).

We sought to verify that the crystals used for NMR and single-crystal XRD crystallized with the same packing. Powder XRD measurements on the same wet microcrystalline samples as used for NMR, in comparison with the single-crystal XRD structure, confirmed that the crystals represent the same phase (Supplementary Fig. 3).

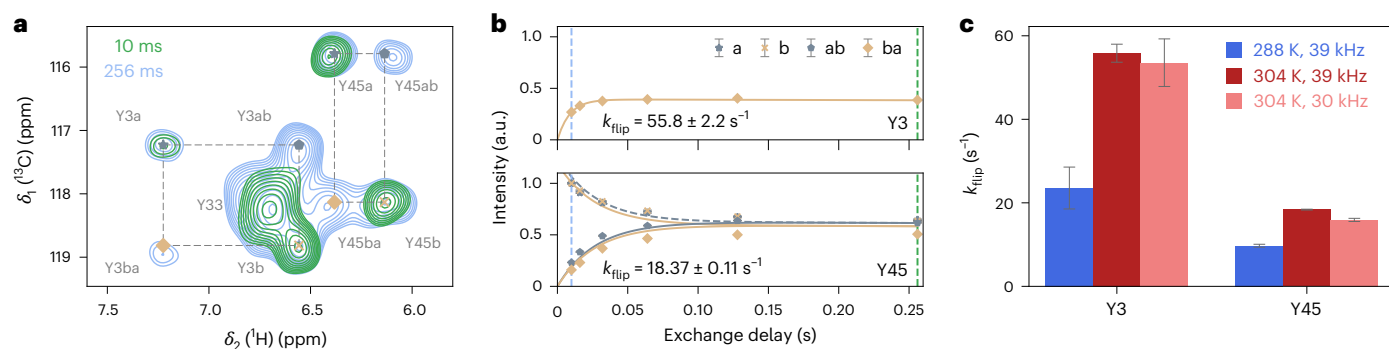
As GB1<sub>QDD</sub> crystals have not been studied previously by MAS NMR, we performed experiments to assign the  $\text{H}^{\text{N}}$ ,  $\text{N}^{\text{H}}$ ,  $\text{C}^{\alpha}$ ,  $\text{C}^{\beta}$  and  $\text{C}^{\gamma}$  resonances (see Methods for details). The quality of the data enabled automatic assignments for most of the protein (Fig. 2a, Supplementary Table 3, and Supplementary Figs. 5 and 6, BMRB access code 53330). We observed two distinct sets of peaks for four residues (Q2, Y3, T18 and V21), which we ascribe to the two non-equivalent molecules. A  $\text{Na}^+$  ion bound in the vicinity of one of the two chains might explain the differences.

Interestingly, signals of residues Q32–D40 were unobservable in the spectra, likely owing to dynamics-induced line broadening (discussed below). Residues D37 and G38 in chain B of the crystal structure could not be modelled owing to blurring of the electron density, and crystallographic B-factors are generally high in this region (Fig. 2b). In previous MAS NMR studies of GB1<sub>T2Q</sub> crystals and IgG-bound GB1<sub>T2Q</sub>, residues Q32–D40 were observable, and B-factors in the GB1<sub>T2Q</sub> crystal

are uniform. These findings suggest crystal-packing-dependent differences in the dynamics of this protein region.

To quantitatively characterize the GB1<sub>QDD</sub> crystal dynamics, we performed  $^{15}\text{N}$  spin-relaxation experiments (longitudinal  $R_1$  relaxation and rotating-frame  $R_{1\rho}$  relaxation at six different spin-lock frequencies) and analysed them using the detectors approach<sup>56–60</sup> (Fig. 2c, green, and Supplementary Figs. 11 and 12). For comparison, we show previously published data for the GB1<sub>T2Q</sub> crystal (grey)<sup>57,58</sup> and the IgG:GB1<sub>T2Q</sub> complex<sup>57,59,60</sup> (yellow). The detectors report on the amount of motion within certain time windows, so-called detector sensitivities  $\rho_i(\tau)$  (Fig. 2c, right; see Methods for details). The position of these windows reflects the timescales to which the measured relaxation rate constants are sensitive. The responses  $\rho_i$  (Fig. 2c, left) report the motional amplitude of each residue in the respective sensitivity  $\rho_i(\tau)$ .

The most striking feature is the elevated  $\mu\text{s}$ – $\text{ms}$  motion for residues 28–31 and 41 (seen as a sharp increase of  $\rho_2$  and  $\rho_3$ ) supporting the conclusion that residues Q32–D40 are broadened owing to enhanced microsecond motions<sup>61</sup>. In GB1<sub>T2Q</sub>, this motion is not observed in crystals (Fig. 2c, grey) or residual-dipolar coupling analyses in solution<sup>62</sup>. While fast motions ( $\rho_1$ ) are similar in the three samples, presumably owing to their local nature, there is an overall increase of  $\rho_1$  and  $\rho_2$  in GB1<sub>QDD</sub> and IgG:GB1, compared with GB1<sub>T2Q</sub>. For IgG:GB1, these dynamics have been ascribed to the overall rocking motion of the protein<sup>63</sup>.



**Fig. 3 | Determination of ring-flip timescales of Y3 and Y45 in crystalline**

**GB1<sub>QDD</sub>.** **a**,  $^1\text{H}$ - $^{13}\text{C}$  EXSY MAS NMR spectrum of crystalline GB1 with 0 ms (green) and 256 ms (light blue) exchange delay at 39 kHz MAS. **b**, Normalized signal intensities depending on the exchange delay and fitted EXSY build-up and decay curves for Y3 and Y45. For Y3, only the build-up of 'ba' was fitted (see text). For

Y45, we performed a combined analysis of all four peaks with  $p_a = p_b = 0.5$  (ref. 66). The errors of the extracted intensities are one standard deviation of the spectral noise level. **c**, Temperature and MAS dependence of  $k_{\text{flip}}$  for Y3 and Y45. Error bars of  $k_{\text{flip}}$  were determined by Monte Carlo error analysis as one standard deviation of 300 iterations (see Methods for details).

### NMR signatures of aromatics in three different states of GB1<sub>QDD</sub>

We obtained high-resolution dipolar-based  $^1\text{H}$ - $^{13}\text{C}$  two-dimensional (2D) MAS NMR spectra of crystalline GB1<sub>QDD</sub> and the IgG:GB1<sub>QDD</sub> complex and compared them with solution-state NMR spectra of GB1<sub>QDD</sub> (Fig. 2d). Experiments connecting ( $^{13}\text{C}^1\text{H}$ ) $^e$  sites to surrounding amide signals (radio frequency-driven recoupling<sup>64</sup>) allowed us to assign all tyrosine signals in the crystal (Supplementary Fig. 7); only one broad, unresolved peak is observed for F30 and F52 (Fig. 2d, lower panel).

The low sensitivity of the IgG:GB1<sub>QDD</sub> sample precluded residue-specific assignments of aromatic signals. We nonetheless confirmed that the Tyr peaks arise from individual residues via  $^{13}\text{C}$ - $^{13}\text{C}$  exchange spectroscopy (EXSY; Supplementary Fig. 10). The absence of cross-peaks indicates that each signal originates from an individual residue, rather than from slow exchange. We, therefore, refer to the three  $\text{Y}^e$  peaks of IgG:GB1 as p1, p2 and p3 in the following.

### Differences in aromatic spectra show altered ring-flip dynamics

The number of signals observed for a ( $^{13}\text{C}^1\text{H}$ ) $^e$  site provides a semi-quantitative view of the ring-flip rate constant  $k_{\text{flip}}$  (Fig. 1b). For GB1<sub>QDD</sub> in solution, each aromatic residue gives rise to one averaged signal because  $k_{\text{flip}} > \Delta\omega$ , where  $\Delta\omega$  is the chemical shift difference between (CH) $^e$ 1 and (CH) $^e$ 2 (Fig. 2d, purple).  $k_{\text{flip}}$  is a few tens of thousands per second for the buried rings, determined previously by relaxation-dispersion experiments at 298 K (Y3,  $(13,700 \pm 1,600) \text{ s}^{-1}$ ; Y45,  $(42,100 \pm 21,700) \text{ s}^{-1}$ ; F30,  $(29,600 \pm 1,500) \text{ s}^{-1}$ ; F52,  $(34,400 \pm 4,500) \text{ s}^{-1}$ )<sup>49</sup>. The solvent-exposed Y33 flips too rapidly for quantification by solution NMR relaxation-dispersion experiments, implying that  $k_{\text{flip}} \gtrsim 100,000 \text{ s}^{-1}$ .

Similar to the situation in solution, a single set of peaks is observed in the IgG:GB1 complex (Fig. 2d, yellow).

The spectrum of the crystalline sample exhibits a very different signature (Fig. 2d, green). Y3 and Y45 each show two peaks split equidistantly around their position in solution, suggesting slower flips in the crystal than in solution, with  $k_{\text{flip}} < \Delta\omega$ . The solvent-exposed Y33 exhibits a single signal indicating fast flips. In contrast to solution, the phenylalanine region shows one broad signal pointing to  $\mu\text{s}$  motion of F30 and/or F52 in crystals.

### Crystal packing slows flips of buried tyrosine rings

To quantify  $k_{\text{flip}}$  of Y3<sup>crystal</sup> and Y45<sup>crystal</sup>, we performed  $^1\text{H}$ - $^{13}\text{C}$  EXSY measurements<sup>65</sup> (Fig. 3). This type of experiment probes the kinetics of conformational exchange via the respective build-up and decay of cross and diagonal peaks during an increasing exchange delay,  $\tau_{\text{ex}}$ . Even though the symmetric labelling results in equally populated

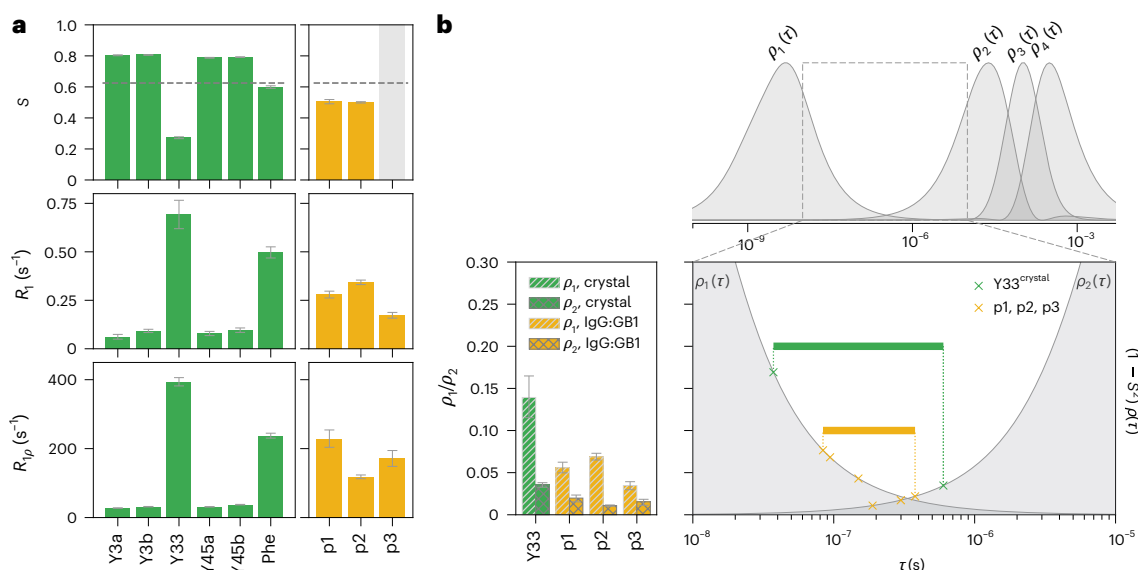
states with  $p_a = p_b = 0.5$ , the intensities of Y3a and Y3b and Y3ab and Y3ba differ (Fig. 3a and Supplementary Fig. 13). Reasons for this could be partial peak overlap with Y33, or differences in  $^1\text{H}$  relaxation and cross-polarization efficiency between the two sites. We therefore only fitted Y3ba for this residue (Fig. 3b). For Y45, we performed a combined fit of all four signals<sup>66</sup>. The extracted flip rates for Y3 and Y45 are  $(55.8 \pm 2.2) \text{ s}^{-1}$  and  $(18.37 \pm 0.11) \text{ s}^{-1}$  at 304 K, about 3 orders of magnitude slower than in solution.

To ensure that the cross-peaks are not a result of magnetization transfer (spin diffusion), we performed experiments at different MAS frequencies and temperatures (Fig. 3c and Supplementary Fig. 13). Magnetization transfer in MAS NMR—which would occur even in static systems<sup>67</sup>—depends on the MAS frequency, whereas ring-flip dynamics are expected to depend on temperature. The observed build-up rate constants are found to only depend on temperature, confirming that the observed process is indeed ring-flip dynamics (see Methods for details).

### Probing fast-flipping rings by MAS NMR methods

EXSY is not applicable to fast-flipping rings that show only a single averaged signal for the two ( $^{13}\text{C}^1\text{H}$ ) $^e$  sites. To gain information on the dynamics of these rings, we measured dipolar order parameters  $S$  and  $^{13}\text{C}$  spin relaxation (Fig. 4). Order parameters range from 1 for totally rigid to 0 for flexible disordered sites and report on amplitudes of motion on timescales up to hundreds of microseconds<sup>68</sup>. The theoretical value for a ring flip, modelled by simulations, is  $S = 0.625$  (ref. 69); reductions below this value point to additional dynamics, such as librational, backbone or additional side-chain motion (that is, around the  $\chi_1$  angle). Rotational-echo double-resonance (REDOR) experiments (Fig. 4a and Supplementary Figs. 14 and 15) resulted in  $S \approx 0.8$  for Y3 and Y45 in the crystal. The fact that these values exceed the theoretical value confirms that the flips of Y3 and Y45 are slower than the timescale sensed by REDOR, that is, about hundreds of microseconds, in agreement with the EXSY data. By contrast, the order parameters of Y33 and the phenylalanines are lower than the expected value (approximately 0.3 and 0.5, respectively), showing that there must be motion in addition to ring flips, occurring faster than hundreds of microseconds (see Supplementary Note 1 and Methods for details).

Interestingly, the electron density map of the room-temperature crystal structure provides support for such additional motion of the ring axis: it reveals alternate conformations of Y33 with the aromatic ring rotated outwards around  $\chi_1$  (Supplementary Fig. 16). Crystallography alone cannot differentiate between conformations statically locked by the crystal lattice and dynamic interconversion. NMR order parameters and the appearance of only one signal confirm fast motion



**Fig. 4 | Determination of ring-flip timescales of fast-flipping tyrosines in GB1.** **a**, Dipolar order parameters  $S$  (top),  $^{13}\text{C}$   $R_1$  (middle) and  $R_{1\rho}$  (bottom, spin-lock field strength  $\nu_{\text{SL}}$  of 15 kHz) relaxation rate constants of Tyr and Phe residues in crystalline GB1 (left) and in the IgG:GB1 complex (right). The dashed line (upper panel) indicates the value of the expected order parameter for a ring flip  $S = 0.625$  (ref. 69). The order parameter of p3 could not be determined owing to low sensitivity (grey box). Errors of  $S$ ,  $R_1$  and  $R_{1\rho}$  were determined by Monte Carlo error analysis as one standard deviation of 500 iterations based on one standard deviation of the spectral noise level (see Methods for details). **b**, Estimation of

ring-flip rates for tyrosines Y3<sup>crystal</sup>, p1, p2 and p3 (see also Supplementary Note 1 and Supplementary Fig. 20). The upper panel shows the detector sensitivities  $\rho_{1-4}(\tau)$ . The lower left panel shows the responses  $\rho_1$  and  $\rho_2$  for Y3<sup>crystal</sup>, p1, p2 and p3. The lower right panel shows a close-up of the area indicated by a dashed box with sensitivities scaled by  $(1 - S^2)$  ( $S = 0.625$ ). Crosses mark the intersection between the responses and the scaled sensitivities. Horizontal bars indicate the range of timescales for the ring flips. Error bars of the responses represent the 5–95% interpercentile range for Monte Carlo simulations with 200 iterations.

(sub-100  $\mu\text{s}$ ) around both  $\chi_1$  and  $\chi_2$  angles of Y33 in the crystal. Only this combined analysis can explain the observations from both methods and yield a comprehensive picture.

To quantify the ring-flip timescales more precisely, we used  $^{13}\text{C}$  spin-relaxation experiments ( $R_1$  and  $R_{1\rho}$  at six different spin-lock frequencies). The markedly different relaxation behaviour of Y33, compared with the slowly flipping Y3 and Y45, indicates faster dynamics (Fig. 4a). We used the detectors approach<sup>56,57</sup> to determine the amplitude of motion within each of the four detector sensitivities  $\rho_{1-4}$  (Fig. 4b, upper panel, and Supplementary Figs. 17, 18 and 20). The theoretically expected response for a ring flip allowed us to define the upper and lower bounds of the ring-flip timescale for Y33<sup>crystal</sup>, which are 37 ns and 600 ns, respectively (Fig. 4b, lower panels, and Supplementary Fig. 8; see Supplementary Note 1 and Methods for details).

The broad Phe signal (F30 and/or F52) has an order parameter on the order of the expected value, which points to ring flips shorter than hundreds of microseconds. The high  $R_1$  and  $R_{1\rho}$  relaxation rate constants correspond to a ns– $\mu\text{s}$  timescale, consistent with the large line width. Extracting site-specific information for F30 and F52 is not possible owing to signal overlap; therefore, we focus on the tyrosines in the following discussion.

### Ring flips in IgG:GB1<sub>QDD</sub> are faster than in crystals

The  $^1\text{H}$ – $^{13}\text{C}$  2D MAS NMR spectra suggest different ring-flip dynamics in IgG:GB1<sub>QDD</sub> than in the crystal. The observation of one peak per tyrosine points to fast ring flips (Fig. 2d) supported by low dipolar order parameters and elevated  $^{13}\text{C}$   $R_1$  and  $R_{1\rho}$  relaxation rate constants (Fig. 4a and Supplementary Fig. 19). The detectors analysis of these relaxation data (see above) resulted in a timescale estimate of the ring flips between tens and hundreds of nanoseconds (Fig. 4b and Supplementary Fig. 20) and is similar for all three Tyr peaks (p1, p2 and p3). Despite the lack of residue-specific assignments, we can conclude that the tyrosines in IgG:GB1<sub>QDD</sub> flip about five orders of magnitude faster than in the crystal.

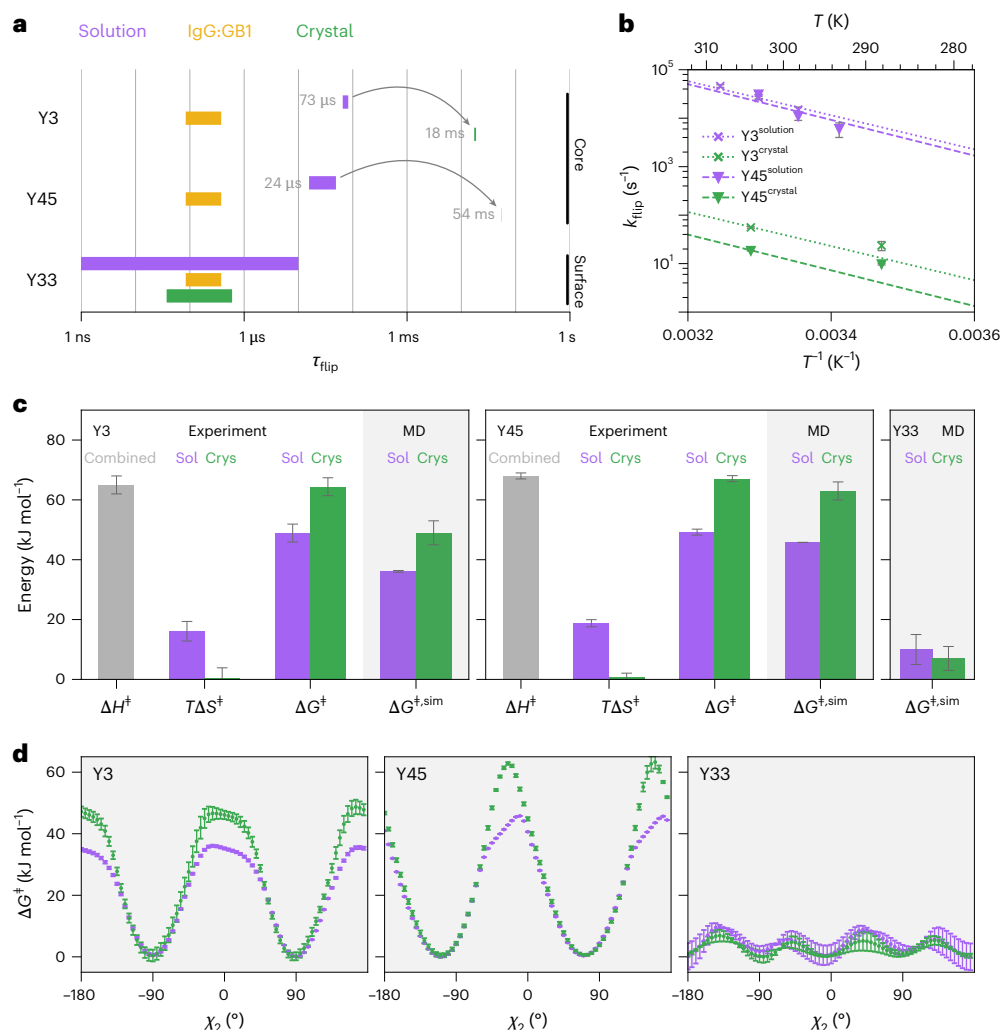
### Ring flips in crystals are less entropically favoured

The ring-flip correlation times  $\tau_{\text{flip}}$  of the tyrosines in all three states of GB1<sub>QDD</sub> are summarized in Fig. 5a. We sought to characterize properties of the transition state of the ring flips by studying the temperature dependence of  $k_{\text{flip}}$  of Y3 and Y45 (Fig. 5b). While the absolute rate constants in solution and crystals are very different, the slopes of as a function of  $1/T$  in the crystal are similar, albeit slightly flatter in crystals than in solution, suggesting that  $\Delta H^{\ddagger, \text{solution}} \geq \Delta H^{\ddagger, \text{crystal}}$  (Supplementary Note 2 and Supplementary Fig. 9). As the temperature range was limited for the MAS NMR measurements, we performed a joint Eyring fit of the rate constants from solution and crystal with the restriction that  $\Delta H^{\ddagger, \text{solution}} = \Delta H^{\ddagger, \text{crystal}}$  for each residue (Fig. 5b). Figure 5c shows the fitted parameters  $\Delta H^{\ddagger}$  and  $T\Delta S^{\ddagger}$  and the resulting Gibbs free energy of activation  $\Delta G^{\ddagger}$  at  $T = 298$  K. We obtain positive values for  $\Delta H^{\ddagger}$  and  $\Delta S^{\ddagger}$  for both residues in solution and the crystal with a large difference in activation entropy,  $\Delta S^{\ddagger, \text{solution}} > \Delta S^{\ddagger, \text{crystal}}$ . This observation confirms that the flips of Y3 and Y45 are slowed down because there is a reduced entropic gain for the ring flip in the crystal compared with solution. Comparing the activation free energies between solution and crystal results in for Y3 and  $(17.9 \pm 1.5)$  kJ mol<sup>−1</sup> for Y45, a value that is useful to benchmark simulations shown below.

Temperature-dependent  $k_{\text{flip}}$  of Y33<sup>crystal</sup> determined with  $^{13}\text{C}$   $R_{1\rho}$  spin relaxation and the detectors analysis did not show temperature dependence within experimental error. This is presumably due to the limited temperature range available for MAS measurements and the uncertainty of the detectors analysis. We therefore refrained from further analysing the temperature-dependent data for Y33.

### MD simulations coincide with experimental ring-flip kinetics

To gain mechanistic insights into the ring flips, we performed enhanced-sampling MD simulations<sup>70</sup>. MD simulations provide atomic-level information on protein dynamics in solution and crystals<sup>31–36</sup> and are highly complementary to experimental methods. With increasing computational power, simulations of large crystal



**Fig. 5 | Thermodynamic parameters of tyrosine ring flips from experiment and simulations.** **a**, Overview of the range of correlation time constants (including uncertainties) at 298 K (solution) or 304 K (crystal and complex). Values for Y33<sup>crystal</sup> (green) and the three tyrosines in the IgG complex (yellow) were obtained from the detectors analysis of <sup>13</sup>C spin relaxation; owing to lack of site-specific assignments, the maximum range of time constants that includes p1, p2 and p3 is shown here. Values for Y3<sup>crystal</sup> and Y45<sup>crystal</sup> were obtained from EXSY. Data for Y3<sup>solution</sup> and Y45<sup>solution</sup> come from <sup>13</sup>C *R*<sub>1ρ</sub> experiments<sup>49</sup>; the upper limit for Y33<sup>solution</sup> flips is obtained from theoretical considerations regarding the limits of solution NMR relaxation dispersion measurements. **b**, Temperature dependence of *k*<sub>flip</sub> of Y3 (crosses) and Y45 (rectangles) in solution<sup>49</sup> (purple) and crystals (green), and the per-residue joint fit (see text). Errors of *k*<sub>flip</sub> were determined by

Monte Carlo error analysis as one standard deviation of 300 iterations based on one standard deviation of the spectral noise level. **c**, Fit results  $\Delta H^\ddagger$  and  $T\Delta S^\ddagger$  from **b** and corresponding activation free energies  $\Delta G^\ddagger$  at *T* = 298 K for Y3 and Y45 in solution (purple) and the crystal (green). Errors were determined by Monte Carlo error analysis as one standard deviation of 300 iterations based on the errors of *k*<sub>flip</sub>.  $\Delta G^\ddagger_{\text{sim}}$  (grey background) are free energies from enhanced-sampling MD simulations (see **d**).  $\Delta H^\ddagger_{\text{solution}} = \Delta H^\ddagger_{\text{crystal}}$  (grey) owing to the fitting constraint. **d**,  $\Delta G^\ddagger$  profiles for ring flips of Y3, Y33 and Y45 obtained from enhanced-sampling MD simulations of GB1 in solution (purple) and the crystal (green). The asymmetry in some profiles is likely due to asymmetric environments of the rings or force field parameters. The error bars are determined by the variance of the free-energy differences measured by different walkers (see Methods for details).

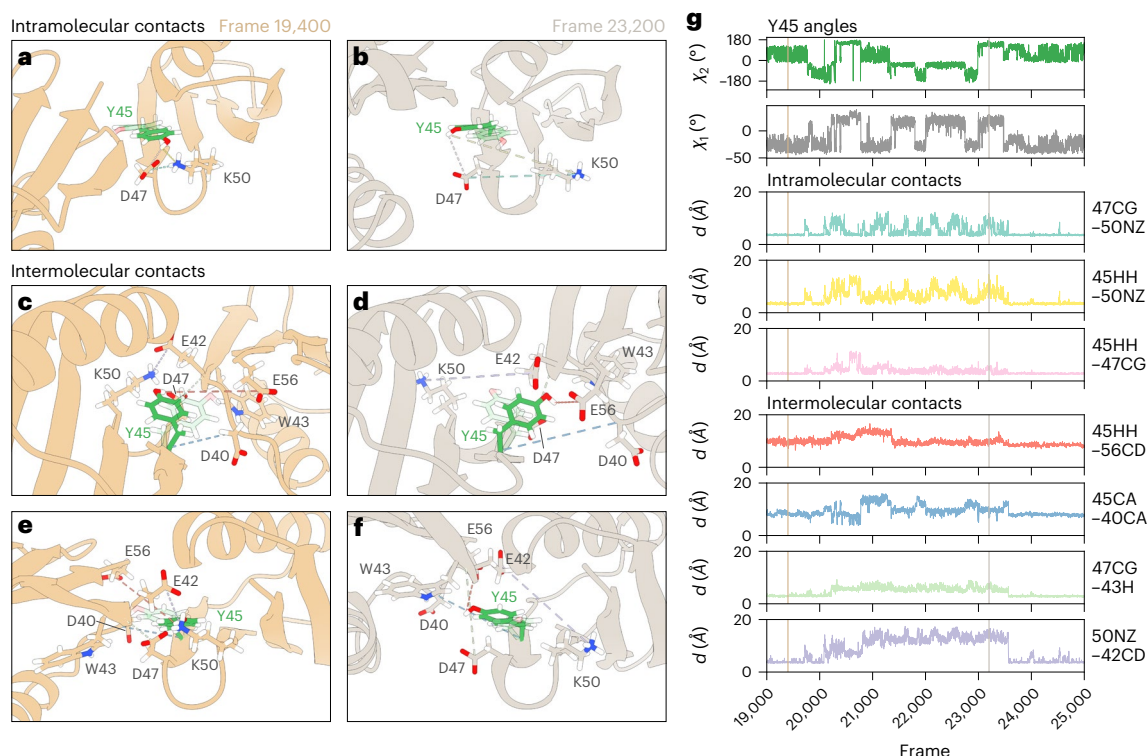
lattices, comprising multiple copies of the unit cell, and extending over the  $\mu$ s timescale, have become feasible<sup>71</sup>. However, the question at hand remains challenging, given that the ring flips occur on timescales spanning from tens of microseconds in solution to tens of milliseconds in the crystal. Such rare events are not amenable to current all-atom equilibrium MD simulations carried out on non-specialized computer architectures<sup>72</sup>.

Enhanced-sampling MD simulations<sup>70</sup> allowed us to accelerate the exploration of the conformational space along the  $\chi_2$  angle, utilizing the well-tempered metadynamics-extended adaptive biasing force (WTM-eABF) algorithm<sup>73</sup> in its multiple-walker variant<sup>74</sup>, as implemented in NAMD<sup>75</sup> and Colvars<sup>76</sup> (see Methods for details). As shown in Fig. 5d, in both aqueous solution and crystal environments, our  $\mu$ s-timescale simulations show that the free-energy barriers against the ring flips of the buried Y3 and Y45 are substantially higher than that

of the more exposed Y33. This result is in quantitative agreement with our experimental observations (Fig. 5c). Importantly, the absolute values of  $\Delta G^\ddagger$  for Y3 and Y45 agree well with those determined experimentally. The values for  $\Delta \Delta G^\ddagger_{\text{sim}}$  are  $(13 \pm 5)$  kJ mol<sup>-1</sup> for Y3 and  $(17 \pm 4)$  kJ mol<sup>-1</sup> for Y45, virtually the same as the ones obtained experimentally ( $\Delta \Delta G^\ddagger_{\text{exp}} = (15 \pm 5)$  kJ mol<sup>-1</sup> for Y3 and  $(17.9 \pm 1.5)$  kJ mol<sup>-1</sup> for Y45). This good quantitative agreement of the free-energy barrier heights between experiments and MD simulations provides a solid foundation for a mechanistic understanding of the ring dynamics.

### Ring dynamics involve a complex network of interactions

In search of mechanisms underlying the ring flips, we investigated potential temporal correlations between ring flips of Y45 (that is,  $\chi_2$ ) and fluctuations in other structural parameters, such as distances and angles.



**Fig. 6 | Structural insights into the interaction network of Y45 in crystals from MD simulations.** **a–f**, Close-up of Y45 and residues interacting with it intramolecularly (**a,b**) or in a neighbouring molecule (**c–f**). Interactions are shown for two different frames of the simulations, representing a state in which Y45 is oriented towards the core of GB1 (frame 19,400; brown; **a,c,e**) or outward rotated (frame 23,200; grey; **b,d,f**). Pale and dark shades of brown and grey

indicate chains A and B of the crystal, respectively. **g**, Time traces of the dihedral angles of the side chain of Y45, and relevant intra- and intermolecular distances. Vertical lines indicate the frames shown in **a–f**. The full time traces and data of the simulation of the monomer (in solution) are shown in Supplementary Figs. 21 and 22.

In the simulation of the GB1 monomer in solution,  $\chi_2$  alternates between two well-defined states (approximately 50° and –130°, respectively). The conformation of the ring axis, observed via the  $\chi_1$  angle, predominantly remains unperturbed and exhibits only rare, short-lived excursions of roughly 50° (Supplementary Fig. 21). There is no apparent temporal correlation between fluctuations in  $\chi_1$  and  $\chi_2$ .

In the crystal lattice,  $\chi_2$  not only fluctuates between two symmetry-equivalent positions separated by 180° but also populates intermediate states. Furthermore, excursions of the ring axis (from  $\chi_1 \approx -25^\circ$  to  $20^\circ$ ) are longer-lived and often coincide with transitions in  $\chi_2$  (Fig. 6g). In the two major  $\chi_1$  states, the ring is rotated more or less ‘outwards’ (Fig. 6a,c,e and Fig. 6b,d,f, respectively). These states are associated with considerable rearrangements in the intramolecular interactions involving Y<sub>45</sub><sup>HH</sup>, D<sub>47</sub><sup>CG</sup> and K<sub>50</sub><sup>NZ</sup>. However, as these interactions are intramolecular, they may also occur in solution and thus cannot explain the observed deceleration of ring flips in the crystal.

We identified an additional set of intermolecular contacts, including residues D40, E42, W43 and E56 from a neighbouring protein subunit in the lattice (Fig. 6c–g). Fluctuations in the distances between these residues and the hydroxyl group of Y45—or its nearby interaction partners—tend to coincide with fluctuations in  $\chi_1$  and/or  $\chi_2$  of Y45. Since these contacts are necessarily absent in solution, we propose that they contribute to the increased free-energy barrier for ring flips observed in the crystal environment.

Overall, MD simulations indicate that a complex network of interactions modulates ring dynamics. We could not find indicators for a coupling between the ring flip and an expansion or contraction of the hydrophobic core (global ‘breathing’ motions; Supplementary Figs. 21–23). Instead, the ring dynamics (both flips and ring-axis reorientation) depend on a subtle interplay of intra- and intermolecular contacts.

## Discussion

Proteins function by sampling a spectrum of conformations. Consequently, they operate within a delicate balance of interactions, and their three-dimensional (3D) structures are typically only marginally stable. Even GB1<sub>QDD</sub>, considered a highly stable protein, has a free energy of unfolding ( $\Delta G^0 \approx 20 \text{ kJ mol}^{-1}$ )<sup>48</sup> that corresponds to the energy of just a few hydrogen bonds. It is, therefore, not surprising that additional interactions with the environment can profoundly reshape the protein’s free-energy landscape. In this study, we used aromatic ring flips as sensitive reporters of rare, cooperative motions to investigate how interactions between GB1 and either neighbouring molecules in the crystal or a large binding partner (the IgG antibody) influence its dynamics compared with the protein in solution.

We observed a spectrum of behaviours for the tyrosine ring flips in GB1. Y33, located on the protein surface, appears largely unaffected by its environment. It undergoes rapid ring flips on the nanosecond time-scale in all three examined states. This finding aligns with a recent study of phenylalanine flips in three different crystal lattices of ubiquitin<sup>77</sup>.

In sharp contrast, the ring flips of Y3 and Y45 in GB1<sub>QDD</sub> are slowed by approximately three orders of magnitude in the crystal. Our thermodynamic analysis reveals that this is due to a change of the entropic barrier between solution and crystal with  $0 < \Delta S^{\ddagger, \text{crystal}} < \Delta S^{\ddagger, \text{solution}}$  (Fig. 5c). This entropic gain is counteracted by an enthalpic penalty, possibly due to a loosened transition state caused by breaking interactions, leading to the observed free-energy barriers  $\Delta G^{\ddagger}$ .  $\Delta G^{\ddagger, \text{crystal}}$  is higher than in solution because  $\Delta S^{\ddagger, \text{crystal}}$  for going from the ring-flip-incompetent state to the ring-flip-competent states is smaller. Contacts with neighbouring molecules might reduce the capacity of GB1 to sample conformations that enable ring flips in the crystal (Fig. 1e). The network of contacts revealed in our MD simulations (Fig. 6c–f) may constitute such

a framework where fewer microstates are accessible within a reduced configuration-space volume owing to defined interactions.

Surprisingly, in the IgG complex, the ring flips of Y3 and Y45 are accelerated compared with solution. We hypothesize that interactions between GB1<sub>QDD</sub> and IgG stabilize conformations that favour ring flipping. This may be facilitated by the fact that GB1–IgG contacts occur primarily on the side of the protein opposite to Y3 and Y45 (Fig. 1f). The low-amplitude overall rocking motion of GB1 within the complex<sup>63</sup>, where contacts between GB1 and IgG are transiently formed and broken, might also contribute to the increased ring-flip rate constants. (Note that we imply this forming and breaking of transient contacts, but they have not actually been shown experimentally.)

The phenylalanine residues in the aromatic cluster (F30 and F52) show markedly different (much faster) ring-flip dynamics in the crystal compared with Y3 and Y45 (Fig. 4a). This observation directly shows that in crystals, the mechanisms enabling F30 and F52 flips must differ from those governing tyrosine rotations, and that, therefore, ring flips in crystalline GB1<sub>QDD</sub> cannot be described by a global ‘breathing motion’.

In solution, by contrast, all aromatics in the core (Y3, Y45, F30 and F52) flip with a similar rate constant in the range of a few tens of thousands per second. One could assume that aromatic ring flips in solution are linked to collective ‘breathing motions’, while this motion is quenched in crystals. However, high-pressure NMR measurements imply that alternative pathways also exist in solution, allowing rings to flip via more local motions and rearrangements without an increase in the total protein volume at the transition state ( $\Delta V^\ddagger$ )<sup>50</sup>. We argue that what is implied by the term ‘breathing motion’ is likely a complex rearrangement of local interactions, not necessarily involving an overall transient expansion.

Recent data support that ring-flip dynamics may not be governed by a single global mode: Lu et al. used fluorine-labelled tyrosines in conjunction with solution-state <sup>19</sup>F NMR to study ring flips in GB1<sub>T2Q</sub> in solution, crowded environments and inside cells<sup>78</sup>. As fluorocarbons are more hydrophobic than hydrocarbons, fluorinated tyrosine rings are expected to engage in stronger local interactions, resulting in higher energy barriers for ring flipping. Consistent with this, ring flips of fluorinated Y45 are slower than those of the non-fluorinated counterpart. Flips of fluorinated Y3 are much faster than those of fluorinated Y45, showing that Y3 and Y45 do not flip synchronously in F-Tyr-labelled GB1<sub>T2Q</sub>. While this behaviour may be due to the unique properties of fluorinated tyrosines, it underscores that timescales of aromatic ring flips are governed by multiple factors that likely involve a combination of local and collective motions.

Our data highlight the complexity of molecular mechanisms, such as protein–protein interactions, that modulate internal dynamics: intermolecular contacts can slow ring flips down (as seen for Y3 and Y45 in crystals), but they may also help stabilize states that facilitate ring flips, as evidenced by the accelerated flips in the IgG:GB1 complex. It is tempting to extrapolate our findings on GB1 to other crystalline systems. Since crystalline environments are inherently more densely packed than the protein’s solution environment, ring flips in many crystals are likely slower than in solution. However, the specifics may depend heavily on context, as demonstrated by our IgG:GB1 results. If intermolecular contacts favour conformations that facilitate ring flips, the crystalline environment might even accelerate them. Even the specific crystal lattice might manipulate ring-flip dynamics, just like overall rocking motion, which also depends on packing density<sup>35</sup>. Our findings may also have relevance for dynamics in the crowded cellular environment; in-cell NMR studies have shown that quinary interactions can promote partially unfolded states<sup>79</sup>, including the case of GB1<sup>80,81</sup>.

In conclusion, our study demonstrates that intermolecular contacts can profoundly influence the timescale of internal protein dynamics. While the precise impact of the crystal lattice or complex certainly depends on the specific protein, our findings underscore that the protein free-energy landscape is complex and highly dependent on the surroundings.

## Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41557-026-02155-0>.

## References

1. RCSB Protein Data Bank. <https://www.rcsb.org/> (accessed 3 March 2025).
2. Weber, G. Energetics of ligand binding to proteins. *Adv. Protein Chem.* **29**, 1–83 (1975).
3. Furnham, N., Blundell, T. L., Depristo, M. A. & Terwilliger, T. C. Is one solution good enough? *Nat. Struct. Mol. Biol.* **13**, 184–185 (2006).
4. Van Den Bedem, H. & Fraser, J. S. Integrative, dynamic structural biology at atomic resolution—it’s about time. *Nat. Methods* **12**, 307–318 (2015).
5. Rosenberg, A. A., Marx, A. & Bronstein, A. M. A dataset of alternately located segments in protein crystal structures. *Sci. Data* **11**, 783 (2024).
6. Frauenfelder, H., Petsko, G. A. & Tsernoglou, D. Temperature-dependent X-ray diffraction as a probe of protein structural dynamics. *Nature* **280**, 558–563 (1979).
7. Artymiuk, P. J. et al. Crystallographic studies of the dynamic properties of lysozyme. *Nature* **280**, 563–568 (1979).
8. Spilburg, C. A., Bethune, J. L. & Vallee, B. L. Kinetic properties of crystalline enzymes. Carboxypeptidase A. *Biochemistry* **16**, 1142–1150 (1977).
9. Doscher, M. S. & Richards, F. M. The activity of an enzyme in the crystalline state: ribonuclease S. *J. Biol. Chem.* **238**, 2399–2406 (1963).
10. Stoddard, B. L. & Farber, G. K. Direct measurement of reactivity in the protein crystal by steady-state kinetic studies. *Structure* **3**, 991–996 (1995).
11. Schlichting, I. in *Protein-Ligand Interactions Methods and Applications* (ed. Nienhaus, G. U.) 155–165 (Humana Press, 2005).
12. Makinen, M. W. & Fink, A. L. Reactivity and cryoenzymology of enzymes in the crystalline state. *Annu. Rev. Biophys. Bioeng.* **6**, 301–343 (1977).
13. Pearson, A. R., Mozzarelli, A. & Rossi, G. L. Microspectrophotometry for structural enzymology. *Curr. Opin. Struct. Biol.* **14**, 656–662 (2004).
14. Pearson, A. R. & Owen, R. L. Combining X-ray crystallography and single-crystal spectroscopy to probe enzyme mechanisms. *Biochem. Soc. Trans.* **37**, 378–381 (2009).
15. Noble, R. W. et al. Correlation of protein functional properties in the crystal and in solution: the case study of T-state hemoglobin. *Protein Sci.* **11**, 1845–1849 (2002).
16. Zhang, X. J., Wozniak, J. A. & Matthews, B. W. Protein flexibility and adaptability seen in 25 crystal forms of T4 lysozyme. *J. Mol. Biol.* **250**, 527–552 (1995).
17. Eyal, E., Gerzon, S., Potapov, V., Edelman, M. & Sobolev, V. The limit of accuracy of protein modeling: influence of crystal packing on protein structure. *J. Mol. Biol.* **351**, 431–442 (2005).
18. Rapp, C. S. & Pollack, R. M. Crystal packing effects on protein loops. *Proteins* **60**, 103–109 (2005).
19. De Zitter, E., Coquelle, N., Oeser, P., Barends, T. R. M. & Colletier, J. P. Xtrapol8 enables automatic elucidation of low-occupancy intermediate-states in crystallographic studies. *Commun. Biol.* **5**, 640 (2022).
20. Brändén, G. & Neutze, R. Advances and challenges in time-resolved macromolecular crystallography. *Science* **373**, eaba0954 (2021).

21. Pearson, A. R. & Mehrabi, P. Serial synchrotron crystallography for time-resolved structural biology. *Curr. Opin. Struct. Biol.* **65**, 168–174 (2020).
22. Fischer, M. Macromolecular room temperature crystallography. *Q. Rev. Biophys.* **54**, e1 (2021).
23. Fraser, J. S. et al. Accessing protein conformational ensembles using room-temperature X-ray crystallography. *Proc. Natl Acad. Sci. USA* **108**, 16247–16252 (2011).
24. Wilson, M. A. Mapping enzyme landscapes by time-resolved crystallography with synchrotron and X-ray free electron laser light. *Annu. Rev. Biophys.* **51**, 79–98 (2022).
25. Chapman, H. N. et al. Femtosecond X-ray protein nanocrystallography. *Nature* **470**, 73–77 (2011).
26. Nass, K. et al. Structural dynamics in proteins induced by and probed with X-ray free-electron laser pulses. *Nat. Commun.* **11**, 1814 (2020).
27. Schulz, E. C. et al. Probing the modulation of enzyme kinetics by multi-temperature, time-resolved serial crystallography. *Nat. Commun.* **16**, 6553 (2025).
28. Weinert, T. et al. Proton uptake mechanism in bacteriorhodopsin captured by serial synchrotron crystallography. *Science* **364**, 61–65 (2019).
29. Coquelle, N. et al. Chromophore twisting in the excited state of a photoswitchable fluorescent protein captured by time-resolved serial femtosecond crystallography. *Nat. Chem.* **10**, 31–37 (2018).
30. Barends, T. R. M., Stauch, B., Cherezov, V. & Schlichting, I. Serial femtosecond crystallography. *Nat. Rev. Methods Primers* **2**, 59 (2022).
31. van Gunsteren, W. F. & Karplus, M. Protein dynamics in solution and in a crystalline environment: a molecular dynamics study. *Biochemistry* **21**, 2259–2274 (1982).
32. Hinsen, K. Structural flexibility in proteins: impact of the crystal environment. *Bioinformatics* **24**, 521–528 (2008).
33. Mollica, L. et al. Atomic-resolution structural dynamics in crystalline proteins from NMR and molecular simulation. *J. Phys. Chem. Lett.* **3**, 3657–3662 (2012).
34. Wall, M. E. et al. Conformational dynamics of a crystalline protein from microsecond-scale molecular dynamics simulations and diffuse X-ray scattering. *Proc. Natl Acad. Sci. USA* **111**, 17887–17892 (2014).
35. Ma, P. et al. Observing the overall rocking motion of a protein in a crystal. *Nat. Commun.* **6**, 8361 (2015).
36. Meisburger, S. P., Case, D. A. & Ando, N. Diffuse X-ray scattering from correlated motions in a protein crystal. *Nat. Commun.* **11**, 1271 (2020).
37. Cerutti, D. S. & Case, D. A. Molecular dynamics simulations of macromolecular crystals. *Wiley Interdiscip. Rev. Comput. Mol. Sci.* **9**, e1402 (2019).
38. Haller, J. D. & Schanda, P. Amplitudes and time scales of picosecond-to-microsecond motion in proteins studied by solid-state NMR: a critical evaluation of experimental approaches and application to crystalline ubiquitin. *J. Biomol. NMR* **57**, 263–280 (2013).
39. Agarwal, V., Xue, Y., Reif, B. & Skrynnikov, N. R. Protein side-chain dynamics as observed by solution- and solid-state NMR spectroscopy: a similarity revealed. *J. Am. Chem. Soc.* **130**, 16611–16621 (2008).
40. Kurauskas, V. et al. Slow conformational exchange and overall rocking motion in ubiquitin protein crystals. *Nat. Commun.* <https://doi.org/10.1038/s41467-017-00165-8> (2017).
41. Tollinger, M., Sivertsen, A. C., Meier, B. H., Ernst, M. & Schanda, P. Site-resolved measurement of microsecond-to-millisecond conformational-exchange processes in proteins by solid-state NMR spectroscopy. *J. Am. Chem. Soc.* **134**, 14800–14807 (2012).
42. Wüthrich, K. & Wagner, G. Internal motion in globular proteins. *Trends Biochem. Sci.* **3**, 227–230 (1978).
43. Snyder, G. H., Rowan, R., Karplus, S. & Sykes, B. D. Complete tyrosine assignments in the high field  $^1\text{H}$  nuclear magnetic resonance spectrum of the bovine pancreatic trypsin inhibitor. *Biochemistry* **14**, 3765–3777 (1975).
44. Campbell, I. D., Dobson, C. M. & Williams, R. J. P. Proton magnetic resonance studies of the tyrosine residues of hen lysozyme assignment and detection of conformational mobility. *Proc. R. Soc. B* **189**, 503–509 (1975).
45. Mariño Pérez, L. et al. Visualizing protein breathing motions associated with aromatic ring flipping. *Nature* **602**, 695–700 (2022).
46. Akke, M. & Weininger, U. NMR studies of aromatic ring flips to probe conformational fluctuations in proteins. *J. Phys. Chem. B* **127**, 591–599 (2023).
47. Lichteneker, R. J., Weinhäupl, K., Schmid, W. & Konrat, R.  $\alpha$ -Ketoacids as precursors for phenylalanine and tyrosine labelling in cell-based protein overexpression. *J. Biomol. NMR* **57**, 327–331 (2013).
48. Lindman, S. et al. Salting the charged surface: pH and salt dependence of protein GB1 stability. *Biophys. J.* **90**, 2911–2921 (2006).
49. Dreydoppel, M., Raum, H. N. & Weininger, U. Slow ring flips in aromatic cluster of GB1 studied by aromatic  $^{13}\text{C}$  relaxation dispersion methods. *J. Biomol. NMR* **74**, 183–191 (2020).
50. Dreydoppel, M., Dorn, B., Modig, K., Akke, M. & Weininger, U. Transition-state compressibility and activation volume of transient protein conformational fluctuations. *JACS Au* **1**, 833–842 (2021).
51. Schmidt, H. L. F. et al. Crystal polymorphism of protein GB1 examined by solid-state NMR spectroscopy and X-ray diffraction. *J. Phys. Chem. B* **111**, 14362–14369 (2007).
52. Nadaud, P. S., Helmus, J. J., Höfer, N. & Jaroniec, C. P. Long-range structural restraints in spin-labeled proteins probed by solid-state nuclear magnetic resonance spectroscopy. *J. Am. Chem. Soc.* **129**, 7502–7503 (2007).
53. Lamley, J. M. et al. Solid-state NMR of a protein in a precipitated complex with a full-length antibody. *J. Am. Chem. Soc.* **136**, 16800–16806 (2014).
54. Sauer-Eriksson, A. E., Kleywegt, G. J., Uhlén, M. & Jones, T. A. Crystal structure of the C2 fragment of streptococcal protein G in complex with the Fc domain of human IgG. *Structure* **3**, 265–278 (1995).
55. Derrick, J. P. & Wigley, D. B. The third IgG-binding domain from streptococcal protein G: an analysis by X-ray crystallography of the structure alone and in a complex with Fab. *J. Mol. Biol.* **243**, 906–918 (1994).
56. Smith, A. A., Ernst, M. & Meier, B. H. Optimized ‘detectors’ for dynamics analysis in solid-state NMR. *J. Chem. Phys.* <https://doi.org/10.1063/1.5013316> (2018).
57. Tatman, B. P. *The Development of Methods to Study Structure and Dynamics in Biological Systems Using Solid-State NMR*. PhD thesis, Univ. Warwick (2023).
58. Stevens, R. A. *The Development of Solid-State NMR Methodology to Study the Dynamics of Proteins and Ice*. PhD thesis, Univ. Warwick (2018).
59. Öster, C. et al. Characterization of protein-protein interfaces in large complexes by solid-state NMR solvent paramagnetic relaxation enhancements. *J. Am. Chem. Soc.* **139**, 12165–12174 (2017).
60. Öster, C., Kosol, S. & Lewandowski, J. R. Quantifying microsecond exchange in large protein complexes with accelerated relaxation dispersion experiments in the solid state. *Sci. Rep.* **9**, 11082 (2019).

61. Schanda, P. & Ernst, M. Studying dynamics by magic-angle spinning solid-state NMR spectroscopy: principles and applications to biomolecules. *Prog. Nucl. Magn. Reson. Spectrosc.* **96**, 1–46 (2016).
62. Markwick, P. R. L., Bouvignies, G. & Blackledge, M. Exploring multiple timescale motions in protein GB3 using accelerated molecular dynamics and NMR spectroscopy. *J. Am. Chem. Soc.* **129**, 4724–4730 (2007).
63. Lamley, J. M., Öster, C., Stevens, R. A. & Lewandowski, J. R. Intermolecular interactions and protein dynamics by solid-state NMR spectroscopy. *Angew. Chem. Int. Ed.* **54**, 15594–15598 (2015).
64. Bennett, A. E. et al. Homonuclear radio frequency-driven recoupling in rotating solids. *J. Chem. Phys.* **108**, 9463–9479 (1998).
65. Meier, B. H. & Ernst, R. R. Elucidation of chemical exchange networks by two-dimensional NMR spectroscopy: the heptamethylbenzenonium ion. *J. Am. Chem. Soc.* **101**, 6441–6442 (1979).
66. Farrow, N. A., Zhang, O., Forman-Kay, J. D. & Kay, L. E. A heteronuclear correlation experiment for simultaneous determination of  $^{15}\text{N}$  longitudinal decay and chemical exchange rates of systems in slow equilibrium. *J. Biomol. NMR* **4**, 727–734 (1994).
67. Tatman, B. P., Franks, W. T., Brown, S. P. & Lewandowski, J. R. Nuclear spin diffusion under fast magic-angle spinning in solid-state NMR. *J. Chem. Phys.* **158**, 184201 (2023).
68. Aebischer, K., Becker, L. M., Schanda, P. & Ernst, M. Evaluating the motional timescales contributing to averaged anisotropic interactions in MAS solid-state NMR. *Magn. Reson.* **5**, 69–86 (2024).
69. Gauto, D. F. et al. Aromatic ring dynamics, thermal activation, and transient conformations of a 468 kDa enzyme by specific  $^1\text{H}$ - $^{13}\text{C}$  labeling and fast magic-angle spinning NMR. *J. Am. Chem. Soc.* **141**, 11183–11195 (2019).
70. Chen, H. & Chipot, C. Enhancing sampling with free-energy calculations. *Curr. Opin. Struct. Biol.* **77**, 102497 (2022).
71. Klyshko, E. et al. Functional protein dynamics in a crystal. *Biophys. J.* **123**, 3244 (2024).
72. Chipot, C. Free energy methods for the description of molecular processes. *Annu. Rev. Biophys.* **52**, 113–138 (2023).
73. Fu, H., Shao, X., Cai, W. & Chipot, C. Taming rugged free energy landscapes using an average force. *Acc. Chem. Res.* <https://doi.org/10.1021/acs.accounts.9b00473> (2019).
74. Comer, J., Phillips, J. C., Schulten, K. & Chipot, C. Multiple-replica strategies for free-energy calculations in NAMD: multiple-walker adaptive biasing force and walker selection rules. *J. Chem. Theory Comput.* **10**, 5276–5285 (2014).
75. Phillips, J. C. et al. Scalable molecular dynamics on CPU and GPU architectures with NAMD. *J. Chem. Phys.* **153**, 2446–2450 (2020).
76. Fiorin, G. et al. Expanded functionality and portability for the Colvars library. *J. Phys. Chem. B* **128**, 11108–11123 (2024).
77. Gauto, D. F. et al. Aromatic ring flips in differently packed ubiquitin protein crystals from MAS NMR and MD. *J. Struct. Biol. X* **7**, 100079 (2023).
78. Lu, M. et al. Characterizing tyrosine ring flips in proteins by  $^{19}\text{F}$  NMR. Preprint at *bioRxiv* <https://doi.org/10.1101/2024.12.29.630691> (2024).
79. Theillet, F. X. & Luchinat, E. In-cell NMR: why and how? *Prog. Nucl. Magn. Reson. Spectrosc.* **132–133**, 1–112 (2022).
80. Cohen, R. D., Guseman, A. J. & Pielak, G. J. Intracellular pH modulates quinary structure. *Protein Sci.* <https://doi.org/10.1002/pro.2765> (2015).
81. Monteith W. B. & Pielak G. J. Correction for Monteith and Pielak, Residue level quantification of protein stability in living cells. *Proc. Natl Acad. Sci. USA* <https://doi.org/10.1073/pnas.1521913112> (2015).
82. Barthels, F., Schirmeister, T. & Kersten, C. BANΔIT: B'-factor analysis for drug design and structural biology. *Mol. Inform.* **40**, 2000144 (2021).

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## Methods

### Sample preparation

**Protein production and purification.** GB1 was produced either (1) without isotope labelling for structure determination, (2) with uniform  $^2\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$  labelling for backbone assignment, or (3) with uniform  $^2\text{H}$  and  $^{15}\text{N}$  and site-specific  $^1\text{H}$ – $^{13}\text{C}$  labelling at the (CH) $^\alpha$ -positions of Phe and Tyr residues for studying aromatic ring dynamics. The M9 minimal medium (M9) for isotopic labelling contained 5.25 g l $^{-1}$  Na $_2$ HPO $_4$ , 3 g l $^{-1}$  KH $_2$ PO $_4$ , 0.5 g l $^{-1}$  NaCl, 1 g l $^{-1}$  NH $_4$ Cl, 2 g l $^{-1}$  D-glucose, 1 mM MgSO $_4$ , 0.1 mM CaCl $_2$ , 0.1 mM MnCl $_2$ , 0.05 mM ZnSO $_4$ , 0.1 mM FeCl $_3$ , 1 mg pyridoxine, 1 mg biotine, 1 mg D-pantothenic acid hemicalcium, 1 mg folic acid, 1 mg choline chloride, 1 mg niacinamide, 0.1 mg riboflavin and 5 mg thiamine hydrochloride.

Transformation of the plasmid containing the GB1 gene with the mutations T2Q, N8D and N37D<sup>48</sup> into *Escherichia coli* BL21(DE3) was achieved by a standard heat shock protocol and plated on LB agar containing 100  $\mu\text{g ml}^{-1}$  ampicillin (Amp). In the following, all shaking steps are performed at 37 °C and 200 rpm, all media formulations contain 100  $\mu\text{g ml}^{-1}$  Amp, and cultures are inoculated such that the optical density at 600 nm (OD $_{600}$ ) is 0.2 unless stated otherwise.

For (1), a single colony was used to inoculate an overnight culture of LB medium. The next day, fresh LB medium was inoculated and shaken until it reached an OD $_{600}$  of 0.6 at which point expression was induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). After shaking for 4 h, cells were collected by centrifugation for 15 min at 4 °C and 5,500 rcf. The pellet was either frozen at –20 °C or used for protein purification immediately.

For (2), the initial preculture in LB was followed by 3 precultures in M9 with 0%, 50% and 100% D $_2$ O over the course of 2 days to allow bacteria to adapt to the deuterated medium. The main culture in M9 medium, which was prepared with 100% D $_2$ O, underwent the same procedure as described for (1). Isotope labelling in the final preculture and the main culture was achieved using  $^{15}\text{NH}_4\text{Cl}$  and D- $^{13}\text{C}_6$ - $^2\text{H}_7$ -glucose.

For (3), the same protocol as for (2) was followed up until the main culture except for using D- $^2\text{H}_7$ -glucose. The main culture was shaken until it reached an OD $_{600}$  of 0.6 at which point 100 mg l $^{-1}$  sodium [3,3- $^2\text{H}_2$ ](3,5- $^{13}\text{C}_2$ ; 2,4,6- $^2\text{H}_3$ ) phenyl) pyruvate and 50 mg l $^{-1}$  sodium [3,3- $^2\text{H}_2$ ](3,5- $^{13}\text{C}_2$ ; 2,6- $^2\text{H}_2$ ) 4-hydroxyphenyl) pyruvate<sup>47</sup> were added. After shaking for 1 h, expression was induced with 1 mM IPTG. The culture was shaken at 20 °C and 200 rpm overnight and collected the next morning as described above.

The cell pellet was resuspended in 10 ml purification buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 7.5) and lysis was achieved by a heat shock at 80 °C for 10 min. After 5 min on ice, the suspension was centrifuged for 40 min at 4 °C and 47,850 rcf. The supernatant was loaded onto a RESOURCE Q column (Cytiva) and eluted in purification buffer with a gradient of 0–1 M NaCl in 10 column volumes. The fractions containing GB1 were concentrated with an Amicon Ultra Centrifugal filter with a 3 kDa molecular weight cut-off and size exclusion chromatography was performed on a HiLoad 16/600 Superdex 75 pg column (Cytiva) in crystallization buffer (50 mM sodium phosphate, pH 6.5).

**Crystallization.** Crystallization was achieved by dialysis<sup>83</sup> in a self-made dialysis button consisting of the upper part of a microtube and a dialysis membrane with a 3.5 kDa molecular weight cut-off (SnakeSkin) (Supplementary Fig. 1a). The protein in crystallization buffer was concentrated to 30 mg ml $^{-1}$  and 120  $\mu\text{l}$  was loaded into the cap of the microtube. The dialysis button was closed with the dialysis membrane and placed into 10 ml of reservoir solution (50% 2-methyl-2,4-pentanediol (MPD), 25% isopropanol) (Supplementary Fig. 1b). Dialysis was performed under constant agitation at 4 °C. Microcrystals started to grow after 1–2 days (Supplementary Fig. 1c).

For NMR samples, the microcrystals were filled into a 1.3 mm (approximately 3 mg) or 1.9 mm (approximately 12 mg) (Supplementary Table 2) MAS rotor (Bruker) by centrifugation (10 min at 4,000 rcf) after 7 days.

To obtain crystals for single-crystal XRD, dialysis was performed for a total duration of 2 weeks, after which large rod-shaped crystals appeared.

For the success of crystallization, avoiding Cl $^-$  ions in the crystallization buffer is crucial<sup>51</sup>. We also found that using an ultracentrifuge to transfer the crystals into the NMR rotor often leads to polymorphic samples. Although we did not investigate this systematically, we suspect that the vacuum in this kind of centrifuge leads to increased isopropanol evaporation, and we achieved more consistent results by using a tabletop centrifuge.

**IgG:GB1 complex.** The sample of the IgG:GB1 complex was prepared as described previously<sup>53</sup>. In short, lyophilized IgG from human serum (Sigma-Aldrich I4506) was dissolved in 50 mM sodium phosphate (pH 6.5) to a concentration of 0.15 mM. The antibody was mixed with 0.3 mM GB1 in the same buffer in a volume ratio of 1:1. The complex precipitates upon mixing and was filled into an MAS NMR rotor by centrifugation (overnight at 68,000 rcf).

### Structure determination

For cryogenic structure determination, crystals were collected at 4 °C, mounted on a MiTeGen cryoloop and cooled to 100 K in liquid nitrogen without any additional cryoprotectant. Data collection was performed on the BM07-FIP2 beamline at the European Synchrotron Radiation Facility (ESRF) at 100 K. Two datasets, each comprising 1,800 images, were recorded with 0.2° rotation and 0.1 s exposure per image. The images were collected at an energy of 12.657 keV from 2 different positions on the same crystal (Supplementary Fig. 2a).

For room-temperature structure determination, the crystal was mounted on a MiTeGen crystallization loop directly on the diffractometer at beamline ID30B of the ESRF (Supplementary Fig. 2b). Data were collected at 295 K using an HC-Lab humidity control device (Arinax), with the relative humidity maintained at 99%, which was experimentally determined to be optimal for preventing crystal dehydration. A single dataset comprising 1,800 images was recorded with an oscillation range of 0.2° and an exposure time of 0.01 s per image. Data were collected at an X-ray energy of 13 keV.

All diffraction frames were processed using autoPROC<sup>84</sup>, data were integrated in XDS<sup>85</sup>, and the integrated intensities were scaled and merged in AIMLESS and POINTLESS, as implemented in CCP4<sup>86</sup>. AlphaFold2<sup>87</sup> was used to predict an initial model, which was used for molecular replacement with PHASER<sup>88</sup>. The model was optimized through iterative rounds of model building in COOT<sup>89</sup> and refinement in PHENIX.REFINE<sup>90</sup>. At all stages of the refinement, translation–libration–screw-rotation was applied. Data collection and refinement statistics are shown in Supplementary Table 1. The cryogenic and room-temperature structures and associated structure factor amplitudes were deposited in the Protein Data Bank (PDB) after validation with MolProbity<sup>91</sup> under the PDB codes 9I2I and 9T8Z, respectively.

### Powder diffraction on microcrystals

Samples for powder XRD were prepared as for the NMR experiments and placed inside borosilicate capillaries with a 1 mm outer diameter, a 0.01 mm wall thickness and an 80 mm length (Hilgenberg). The samples were measured on a XEUS 3.0 HR (Xenocs SAS) in <0.1 mbar vacuum using radiation from a microfocus Cu source collimated with a 3D multi-layer mirror and shaped by scatterless slits, and an Eiger2 1M pixel array detector. Several sample-detector distances and collimation settings were tested for optimal data quality. A total of 36 frames of 120 s exposure each from multiple places in a capillary were cumulated to achieve good orientation statistics. The frames were cumulated and reduced in the XSACT suite<sup>92</sup>. No background or buffer subtraction was done since all other scatterers (capillary, buffer) showed featureless patterns.

Analysis of the data was done using GSASII<sup>93</sup>. We refined the unit cell parameters and the sample displacement following the approach

of Le Bail for the peak intensities<sup>94</sup>. An angle-independent Gaussian and a domain size-related Lorentzian term were used to fit the peak shapes and a polynomial background.

All peaks could be indexed with the C121 phase also observed in single-crystal diffraction; no other phase was detectable. Refinement of the parameters resulted in  $a = 77.9 \text{ \AA}$ ,  $b = 35.3 \text{ \AA}$ ,  $c = 51.4 \text{ \AA}$  and  $\beta = 122.3^\circ$ , very close to the values obtained by single-crystal XRD (Supplementary Table 1).

### NMR spectroscopy

MAS NMR experiments were recorded on a Bruker Avance Neo spectrometer operating at 16.44 T (700 MHz  $^1\text{H}$  Larmor frequency) with triple-channel ( $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$ ) probe heads for 1.3 mm or 1.9 mm MAS rotors. All measurements were performed with  $^1\text{H}$  detection and cross-polarization-based transfers. The following sequences used in this work are implemented in the ssNMRlib library<sup>95</sup>: 2D hNH, 3D hCONH, 3D hCOcANH, 3D hCANH, 3D hCAcANH, 3D hcaCBcANH, 3D hcaCBcacoNH, 3D HhNH radio frequency-driven recoupling, pseudo-3D hNHR<sub>1</sub> and pseudo-3D hNHR<sub>1p</sub>. Experiments used to study the aromatic  $^1\text{H}$ – $^{13}\text{C}$  signals of specifically ( $\text{CH}$ ) <sup>$\epsilon$</sup>  labelled Phe and Tyr residues are shown in Supplementary Fig. 4 (not currently implemented in NMRlib). They all contain a  $^{13}\text{C}$  EBURP2 pulse<sup>96</sup> to selectively excite the aromatic C <sup>$\epsilon$</sup>  signal and suppress the natural-abundance carbon background that results particularly from cross-polarization from amide hydrogens (which spectrally overlap with those of aromatic hydrogens) and C <sup>$\alpha$</sup>  or C <sup>$\gamma$</sup>  carbons. In addition, the water suppression element was moved in front of the  $^{13}\text{C}$  chemical-shift evolution period  $t_1$  to avoid cross-peaks between C <sup>$\epsilon 1$</sup>  and C <sup>$\epsilon 2$</sup>  that may arise from exchange dynamics or magnetization transfer (proton-driven spin diffusion). In all cases, the water-suppression element consisted of a composite-pulse decoupling sequence with constant amplitude and a pseudo-random variation of the pulse durations and phases. The determination of the sample temperature was performed on an external sample (1.3 mm or 1.9 mm rotor) containing lyophilized protein rehydrated with a solution of 25 mM TmDOTP and 10 mM 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) in 80% D<sub>2</sub>O for each measurement condition<sup>97</sup>. The experimental conditions are summarized in Supplementary Table 2.

**Assignment experiments.** Six 3D spectra (hCONH, hCOcANH, hCANH, hCAcANH, hcaCBcANH and hcaCBcacoNH) were recorded to assign the resonances of H<sup>N</sup>, N<sup>H</sup>, C <sup>$\gamma$</sup> , C <sup>$\alpha$</sup>  and C <sup>$\beta$</sup>  atoms of GB1 in the crystal.

A 3D HhNH experiment with  $^1\text{H}$ – $^1\text{H}$  radio frequency-driven recoupling was used to assign aromatic (H <sup>$\epsilon$</sup> ) protons of (CH) <sup>$\epsilon$</sup> -labelled Phe and Tyr by probing their proximity to amide (H<sup>N</sup>) protons.

A 2D HcH EXSY experiment was recorded to probe potential exchange between  $^{13}\text{C}$  atoms in tyrosines in the IgG:GB1 complex (Supplementary Fig. 4b). The exchange delay  $\tau$  of 256 ms was applied while magnetization was stored on  $^{13}\text{C}$  nuclei after the indirect  $^1\text{H}$  evolution period.

**Experiments to probe dynamics.** Experiments to probe amide  $^{15}\text{N}$  and aromatic  $^{13}\text{C}$  longitudinal  $R_1$  and rotating frame relaxation  $R_{1\rho}$  were performed as pseudo-3D spectra. The pseudo dimension was used to vary the relaxation delay  $\tau$  or the duration of the spinlock pulse for  $R_1$  and  $R_{1\rho}$ , respectively (Supplementary Fig. 4c,d). The delays and pulse lengths used can be found in Supplementary Figs. 11 and 17–19.

EXSY experiments were performed to study the timescale of tyrosine ring flips for which separate signals for H <sup>$\epsilon 1$</sup> –C <sup>$\epsilon 1$</sup>  (site a) and H <sup>$\epsilon 2$</sup> –C <sup>$\epsilon 2$</sup>  (site b) could be observed (Supplementary Fig. 4e). If a ring flip during the longitudinal C <sup>$\epsilon$</sup>  mixing time  $\tau$ , sites a and b exchange, resulting in the cross-peaks H <sup>$\epsilon 2$</sup> –C <sup>$\epsilon 1$</sup>  (ab) and H <sup>$\epsilon 1$</sup> –C <sup>$\epsilon 2$</sup>  (ba) in the final spectrum. The experiment was implemented as a pseudo-4D experiment with two frequency dimensions ( $^1\text{H}$  and  $^{13}\text{C}$ ), and two dimensions related to the EXSY: along one dimension, a variable exchange delay  $\tau$  allowed monitoring the build-up (owing to the exchange process) and subsequent

decay (owing to  $R_1$  relaxation, that is, incoherent processes, and possibly spin diffusion, that is, coherent transfer of magnetization to other nuclei) of cross-peak intensities. In the fourth dimension, the position of the exchange delay  $\tau$  was alternated, to be either before or after the  $^{13}\text{C}$  chemical-shift evolution period  $t_1$ . In the former case, only diagonal peaks are observed, and their intensity decays over  $\tau$ . In the latter case, the build-up of exchange cross-peaks is observed, and the intensities of diagonal and cross-peaks are subject to the same decay as in the reference experiment. When subtracting the two spectra, diagonal peaks are eliminated, facilitating the quantification of cross-peaks. As part of the exchange delay, an additional water suppression element with a constant duration was implemented, making the minimum possible  $\tau$  10 ms. We verified whether the observed cross-peaks may result from spin diffusion rather than from ring flips, by repeating the experiments at different MAS frequencies  $\nu_r$  (30 kHz and 39 kHz) while keeping the sample temperature constant. Spin diffusion, but not ring-flip dynamics, is expected to be MAS frequency dependent.

REDOR experiments were performed with a shift  $\Delta$  of the first inversion pulse in each rotor period  $\tau_r$  to scale down the dipolar coupling and allow better sampling<sup>98,99</sup> (Supplementary Fig. 4f). The dephasing curve was sampled at time points  $2\tau_r + (n-1)2\tau_r$  with  $n = 1-15$ . The incrementation of rotor periods was implemented as a third dimension. The REDOR block was followed by a short z-filter to suppress unwanted coherences that might have built up during the recoupling period. The reference experiment in which the inversion pulses on the  $^1\text{H}$  channel were replaced with equally long delays was sampled at  $n = 1, 7, 13$ , and the missing reference intensities were obtained from a linear fit of these points. This approach allows spending more time on collecting the more informative data points of the recoupling experiment because the decay in such highly deuterated samples is slow and sensitivity high, such that the fit of the data is very robust.

### NMR data analysis

All spectra were processed with Bruker Topspin 4.1.4 and then converted to UCSF format with the bruk2ucsf programme provided in Sparky<sup>100</sup>. The analysis of the experiments to investigate dynamics was done with scripts using the NmrGlue package for Python<sup>101</sup>. All spectra were indirectly referenced to DSS via MPD<sup>69</sup>.

**Backbone assignment.** Non-uniform sampling reconstruction and processing of backbone assignment spectra was performed in Topspin before conversion to UCSF format. Peaks were picked in CcpNmr Analysis Version 3<sup>102</sup> and exported for automated resonance assignment by FLYA (CYANA v3.98.15)<sup>103</sup>. Chemical-shift tolerances were set to 0.1 ppm for  $^1\text{H}$  and 0.4 ppm for  $^{13}\text{C}$  and  $^{15}\text{N}$  resonances. Minor manual corrections to the automated assignment were made in CcpNmr. Representative sections of the 3D spectra are shown in Supplementary Fig. 5. The completeness of the assignment is visualized in Supplementary Fig. 6, and all assigned chemical shifts can be found in Supplementary Table 3. The full assignment is published under the BMRB access code 53330.

**Determination of ring-flip rates from EXSY experiments.** The pseudo-4D spectrum was processed as individual 2D planes resulting in a reference and an exchange spectrum for each exchange time. Peak intensities were obtained by fitting a 2D Gaussian lineshape with functions provided in the NmrGlue package. Intensities of the diagonal and cross-peaks were extracted in the reference and difference spectrum respectively. For residue Y45, the four signals (A, B, AB and BA) were fitted as described in Farrow et al. (1994)<sup>66</sup> to obtain the rate of the ring flip  $k_{\text{flip}}$ . The longitudinal relaxation rates  $R_{1,A}$  and  $R_{1,B}$  were set to the determined values from the relaxation experiment, and the populations were set to  $p_A = p_B = 0.5$ . Owing to substantial signal overlap of Y3 and Y33, we extracted  $k_{\text{flip}}$  of residue Y3 only from the build-up of the cross-peak BA intensities  $I$  according to  $I(\tau) = I(0)(1 - \exp[-2k_{\text{flip}}\tau]) \exp[-R_{1,B}\tau]$ . The fitted curves are shown in Supplementary Fig. 13. Errors were

determined by Monte Carlo analysis. Five hundred noisy datasets were generated using the spectral noise level defined as the standard deviation of intensities across a spectral region without any signal. The error of each fit parameter was estimated as the standard deviation of that given parameter across the fits of these synthetic datasets.

**Determination of relaxation rate constants.** Peak intensities were extracted from a series of 2D planes with different relaxation delays ( $R_1$ ) or spinlock pulse length ( $R_{1\rho}$ ) as described above. Relaxation rate constants were obtained by an exponential fit of the intensities. Error analysis was performed as described above. All fits are shown in Supplementary Figs. 17–19.

**Detectors.** Relaxation rates were analysed using the detectors approach as implemented in the package Detectorist<sup>67</sup>. Sensitivities were calculated for each of the relaxation rates measured. For the site-selective  $^{13}\text{C}$ – $^1\text{H}$  Tyrs, the sensitivity was calculated assuming a  $^{13}\text{C}$ – $^1\text{H}$  dipolar coupling at a distance of 1.09 Å, and a  $^{13}\text{C}$  CSA tensor with principal components 180 ppm, 143 ppm and 27 ppm (ref. 104). For the backbone  $^{15}\text{N}$  sites, the sensitivity was calculated assuming the directly bonded  $^1\text{H}$  at a distance of 1.02 Å, and principal components of the  $^{15}\text{N}$  CSA tensor of 229.2 ppm, 78.6 ppm and 52.3 ppm (ref. 105). Each sensitivity was digitized using 200 time points, logarithmically spaced between 100 fs  $\text{rad}^{-1}$  and 1 ms  $\text{rad}^{-1}$ . It should be noted that owing to the form of the spectral density, the correlation times involved are naturally in units of seconds per radian. For comparison with results from EXSY, these have been explicitly converted into the base unit of seconds.

Detector optimization was performed using the singular value decomposition method<sup>106</sup>. Specifically, a matrix,  $\mathbf{M}$ , was produced whereby each of the  $N$  rows represents the sensitivity of a corresponding relaxation rate. The singular value decomposition of this matrix was taken:

$$\mathbf{M} = \mathbf{U}\mathbf{\Sigma}\mathbf{V}^T. \quad (1)$$

The singular values (diagonal entries in  $\mathbf{\Sigma}$ ) were sorted, the matrices truncated to include  $k$  singular values ( $k$  was varied from 2 to 7 for model selection). Linear programming was performed to optimize linear combinations of the orthogonal singular vectors in the truncated  $\mathbf{V}_k^T$  to identify well-formed detectors. The coefficients of  $\mathbf{V}_k^T$  giving rise to detectors then form the rows of the transformation matrix  $\mathbf{Q}$ , which can be used to determine the matrix  $\mathbf{r}$ , containing ‘detector vectors’:

$$\mathbf{r} = \mathbf{U}_k \mathbf{\Sigma}_k \mathbf{Q}^{-1}, \quad (2)$$

for which each of the  $N$  columns,  $\mathbf{r}_i$ , gives a linear combination of  $\leq k$  detectors giving the corresponding relaxation rate. The detector responses may then be calculated using a non-negative least squares to solve for the detector responses,  $\mathbf{p}$ :

$$\mathbf{p} = \left[ \begin{array}{c} \mathbf{r}_1/e_1 \\ \vdots \\ \mathbf{r}_N/e_N \end{array} \right]^{-1} \frac{\mathbf{R}}{\mathbf{e}}, \quad (3)$$

where  $\mathbf{R}$  and  $\mathbf{e}$  are vectors containing the experimental relaxation rates and uncertainties, respectively. The detector sensitivities are obtainable directly from the product  $\mathbf{Q}\mathbf{V}_k^T$ . The number of singular values to be included in the analysis was identified by performing the analysis for  $k = 2$ – $7$ , back-calculating the relaxation rates, then choosing the number of singular values that gave the lowest median reduced chi-square value.

**Determination of dipolar order parameters from REDOR experiments.** Peak intensities were extracted as described above. The

reference curve was linearly interpolated to obtain intensities  $S_0$  at the same time points as in the dephasing experiment  $S_{\text{rec}}$ . The REDOR curve was calculated as  $\Delta S/S_0 = (S_0 - S_{\text{rec}})/S_0$ . The observed dipolar coupling strength  $\delta_D^{\text{obs}}$  and tensor asymmetry  $\eta$  were determined by a 2D grid search against simulated curves. The simulated curves with varying  $\delta_D$  (0–50,000 Hz with a step size of 50 Hz) and  $\eta$  (0–1 with a step size of 0.05) were generated using the GAMMA simulation package for numerical spin dynamics simulations<sup>107</sup>. Chi-square minimization between the simulated and experimental curves was used to find the best-fit values for  $\delta$  and  $\eta$ . Error determination was performed as described above. Dipolar order parameters  $S$  were calculated with  $S = (\delta_D^{\text{obs}}/\delta_D^{\text{rigid}})$  with  $\delta_D^{\text{rigid}} = 46,656$  Hz, corresponding to a  $^1\text{H}$ – $^{13}\text{C}$  distance of 1.09 Å. The fits are shown in Supplementary Figs. 14 and 15.

## Computational details

**Computational assays.** Two distinct computational assays were constructed for GB1 in its crystal form and in an aqueous solution, using the visualization package VMD 1.9.4<sup>108</sup>, and the CHARMM-GUI utility<sup>109</sup>. The crystal assay consisted of 8 proteins and 2,456 water molecules. The aqueous solution consisted of a single protein immersed in a bath of 6,384 water molecules. To ensure electric neutrality of each assay and mimic physiological conditions, NaCl was added at a concentration of 150 mM. After suitable thermalization, the dimensions of computational assays were approximately  $a = 80.2$  Å,  $b = 36.4$  Å and  $c = 53.2$  Å, with  $\alpha = 90.0^\circ$ ,  $\beta = 120.7^\circ$  and  $\gamma = 90.0^\circ$ , for the crystal form, and  $a = 58.7$  Å,  $b = 58.7$  Å and  $c = 58.7$  Å, for the aqueous solution, corresponding to 16,704 and 26,432 atoms, respectively.

**MD simulations.** All MD simulations were carried out using NAMD 3.0<sup>75</sup>, with the AMBER ff19SB force field for proteins and the OPC water model<sup>110</sup>. Periodic boundary conditions were applied for all computational systems. The temperature was maintained at 298 K by means of a stochastic velocity rescaling thermostat<sup>111</sup> with a time parameter of 1 ps<sup>−1</sup> for coupling, and pressure at 1 bar, using the Langevin piston algorithm<sup>112</sup>. All covalent bonds between heavy and hydrogen atoms were constrained to their equilibrium length with the Rattle algorithm<sup>113</sup>. Water molecules were constrained to their equilibrium geometry with the Settle algorithm<sup>114</sup>. Long-range electrostatic forces were computed with the particle-mesh Ewald algorithm<sup>115</sup> and a grid spacing of 1.2 Å. Short-range van der Waals and electrostatic interactions were smoothly truncated with a 10 Å spherical cut-off. Hydrogen mass repartitioning was applied to the protein and surrounding lipids to allow longer integration time steps to be utilized<sup>116</sup>. The Verlet-I/r-RESPA multiple time-stepping algorithm was used to integrate the equations of motion with an effective time step of 4 fs for short-range interactions and 8 fs for long-range interactions<sup>117</sup>.

**Free-energy calculations.** As a preamble to the free-energy calculations, the two computational assays underwent a suitable energy minimization, followed by 400 ns of thermalization bereft of geometrical restraints. The potentials of mean force (PMFs),  $\Delta G(\chi_2)$ , underlying the isomerization of the side chain of residues Y3, Y33 and Y45 about the  $\chi_2$  torsional angle, in the crystal form of the protein and in the aqueous solution, were determined with the well-tempered metadynamics extended adaptive biasing force (WTM-eABF) algorithm<sup>73,118</sup>. In a nutshell, it relies upon the integration of the average force acting on  $\chi_2$ , obtained from unconstrained MD simulations. In the course of the simulation, a biasing force is estimated such that, once applied to the system, it yields a Hamiltonian devoid of an average force exerted along  $\chi_2$ . As a result, all values of  $\chi_2$  are sampled with an equal probability, which, in turn, greatly improves the accuracy of the calculated free energies. The transition pathway spanned  $360^\circ$ , that is,  $-180^\circ \leq \chi_2 \leq +180^\circ$ , and was discretized in bins  $5^\circ$  wide, where samples of the local force acting along  $\chi_2$  were accrued. To mitigate the risk of deleterious non-equilibrium effects, no time-dependent bias was applied until a

threshold of 10,000 samples was reached<sup>119</sup>. In consideration of the substantial isomerization timescales measured experimentally, likely to be related to slowly relaxing degrees of freedom coupled to  $\chi_2$ , likely to hamper convergence of the free-energy calculation, a multiple-walker strategy was used to improve ergodic sampling<sup>74</sup>, with up to four walkers. The statistical error associated to the PMFs was estimated from the variance of the free-energy differences measured by each walker. Analysis of the trajectories was performed using VMD 1.9.4<sup>75</sup>.

## Data availability

The cryo and room-temperature crystal structures of GB1<sub>QDD</sub> are deposited at the PDB under the access codes 9I2I and 9T8Z, respectively. The solid-state NMR backbone assignment of GB1<sub>QDD</sub> is deposited at the BMRB under the access code 53330. NMR spectra, analysis scripts and raw data are publicly available at the ISTA research explorer (<https://doi.org/10.15479/AT-ISTA-20641>)<sup>120</sup>. Files to reproduce the enhanced-sampling MD simulations are publicly available at the ISTA research explorer (<https://doi.org/10.15479/AT-ISTA-21145>)<sup>121</sup>.

## References

83. Nadaud, P. S., Helmus, J. J., Kall, S. L. & Jaroniec, C. P. Paramagnetic ions enable tuning of nuclear relaxation rates and provide long-range structural restraints in solid-state NMR of proteins. *J. Am. Chem. Soc.* **131**, 8108–8120 (2009).
84. Vonrhein, C. et al. Data processing and analysis with the autoPROC toolbox. *Acta Crystallogr. D Biol. Crystallogr.* **67**, 293–302 (2011).
85. Kabsch, W. Xds. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 125–132 (2010).
86. Evans, P. R. An introduction to data reduction: space-group determination, scaling and intensity statistics. *Acta Crystallogr. D Biol. Crystallogr.* **67**, 282–292 (2011).
87. Jumper, J. et al. Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583–589 (2021).
88. McCoy, A. J. et al. Phaser crystallographic software. *J. Appl. Crystallogr.* **40**, 658–674 (2007).
89. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 486–501 (2010).
90. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 213–221 (2010).
91. Williams, C. J. et al. MolProbity: more and better reference data for improved all-atom structure validation. *Protein Sci.* **27**, 293–315 (2018).
92. Xenocs. 'XSACT: X-ray scattering analysis and calculation tool.' SAXS & WAXS data analysis software, version 2.10. <https://xenocs.com> (2023).
93. Toby, B. H. & Von Dreele, R. B. GSAS-II: the genesis of a modern open-source all purpose crystallography software package. *J. Appl. Crystallogr.* **46**, 544–549 (2013).
94. Le Bail, A. Whole powder pattern decomposition methods and applications: a retrospection. *Powder Diffr.* **20**, 316–326 (2005).
95. Vallet, A., Favier, A., Brutscher, B. & Schanda, P. ssNMRlib: a comprehensive library and tool box for acquisition of solid-state nuclear magnetic resonance experiments on Bruker spectrometers. *Magn. Reson.* **1**, 331–345 (2020).
96. Geen, H. & Freeman, R. Band-selective radiofrequency pulses. *J. Magn. Reson.* **93**, 93–141 (1991).
97. Zhang, D., Itin, B. & McDermott, A. E. TmDOTP: an NMR-based thermometer for magic angle spinning NMR experiments. *J. Magn. Reson.* **308**, 106574 (2019).
98. Gullion, T. & Schaefer, J. Rotational-echo double-resonance NMR. *J. Magn. Reson.* **81**, 196–200 (1989).
99. Schanda, P., Meier, B. H. & Ernst, M. Quantitative analysis of protein backbone dynamics in microcrystalline ubiquitin by solid-state NMR spectroscopy. *J. Am. Chem. Soc.* **132**, 15957–15967 (2010).
100. Goddard, T. D. & Kneller, D. G. *Sparky 3* (University of California, 2008).
101. Helmus, J. J. & Jaroniec, C. P. NmrGlue: an open source Python package for the analysis of multidimensional NMR data. *J. Biomol. NMR* **55**, 355–367 (2013).
102. Skinner, S. P. et al. CcpNmr AnalysisAssign: a flexible platform for integrated NMR analysis. *J. Biomol. NMR* **66**, 111–124 (2016).
103. Schmidt, E. & Güntert, P. A new algorithm for reliable and general NMR resonance assignment. *J. Am. Chem. Soc.* **134**, 12817–12829 (2012).
104. Ye, C., Fu, R., Hu, J., Hou, L. & Ding, S. Carbon-13 chemical shift anisotropies of solid amino acids. *Magn. Reson. Chem.* **31**, 699–704 (1993).
105. Lamley, J. M. et al. Unraveling the complexity of protein backbone dynamics with combined <sup>13</sup>C and <sup>15</sup>N solid-state NMR relaxation measurements. *Phys. Chem. Chem. Phys.* **17**, 21997–22008 (2015).
106. Smith, A. A., Ernst, M., Meier, B. H. & Ferrage, F. Reducing bias in the analysis of solution-state NMR data with dynamics detectors. *J. Chem. Phys.* **151**, 034102 (2019).
107. Smith, S. A., Levante, T. O., Meier, B. H. & Ernst, R. R. Computer simulations in magnetic resonance. An object-oriented programming approach. *J. Magn. Reson. Ser. A* **106**, 75–105 (1994).
108. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. *J. Mol. Graphics* **14**, 33–38 (1996).
109. Jo, S., Kim, T., Iyer, V. G. & Im, W. CHARMM-GUI: a web-based graphical user interface for CHARMM. *J. Comput. Chem.* **29**, 1859–1865 (2008).
110. Tian, C. et al. ff19SB: amino-acid-specific protein backbone parameters trained against quantum mechanics energy surfaces in solution. *J. Chem. Theory Comput.* **16**, 528–552 (2020).
111. Bussi, G., Donadio, D. & Parrinello, M. Canonical sampling through velocity rescaling. *J. Chem. Phys.* **126**, 14101 (2007).
112. Feller, S. E., Zhang, Y., Pastor, R. W. & Brooks, B. R. Constant pressure molecular dynamics simulation: the Langevin piston method. *J. Chem. Phys.* **103**, 4613–4621 (1995).
113. Andersen, H. C. Rattle: a 'velocity' version of the shake algorithm for molecular dynamics calculations. *J. Comput. Phys.* **52**, 24–34 (1983).
114. Miyamoto, S. & Kollman, P. A. Settle: an analytical version of the SHAKE and RATTLE algorithm for rigid water models. *J. Comput. Chem.* **13**, 952–962 (1992).
115. Darden, T., York, D. & Pedersen, L. Particle mesh Ewald: an  $N$ -log( $N$ ) method for Ewald sums in large systems. *J. Chem. Phys.* **98**, 10089–10092 (1993).
116. Hopkins, C. W., Le Grand, S., Walker, R. C. & Roitberg, A. E. Long-time-step molecular dynamics through hydrogen mass repartitioning. *J. Chem. Theory Comput.* **11**, 1864–1874 (2015).
117. Tuckerman, M., Berne, B. J. & Martyna, G. J. Reversible multiple time scale molecular dynamics. *J. Chem. Phys.* **97**, 1990–2001 (1992).
118. Fu, H. et al. Zooming across the free-energy landscape: shaving barriers, and flooding valleys. *J. Phys. Chem. Lett.* **9**, 4738–4745 (2018).
119. Miao, M. et al. Avoiding non-equilibrium effects in adaptive biasing force calculations. *Mol. Simul.* **47**, 390–394 (2021).
120. Becker, L. M. & Schanda, P. Data for 'Aromatic ring flips reveal reshaping of protein dynamics in crystals and complexes'. <https://doi.org/10.15479/AT-ISTA-20641> (2025).
121. Becker, L. M., Schanda, P. & Chipot, C. Additional data for 'Aromatic ring flips reveal reshaping of protein dynamics in crystals and complexes'. <https://doi.org/10.15479/AT-ISTA-21145> (2026).

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## Author contributions

P.S., L.M.B., C.C. and U.W. designed the project. A.K. prepared the proteins. S.E. determined the crystal structures. D.M.B. collected and analysed the powder XRD data. M.D. and U.W. performed and analysed

the solution NMR experiments and thermodynamic analysis of ring-flip rates. B.P.T. analysed the solid-state NMR relaxation data. H.F. and C.C. performed and analysed the MD simulations. L.M.B. prepared the samples, performed the solid-state NMR experiments, analysed the MD and solid-state NMR data, and prepared the figures. L.M.B., P.S., H.F., U.W. and C.C. wrote the paper with input from all authors.

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## Competing interests

The authors declare no competing interests.

## Additional information

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