Dynamic regulation of GDP binding to G proteins revealed by magnetic field-dependent NMR relaxation analyses

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Heterotrimeric guanine-nucleotide-binding proteins (G proteins) serve as molecular switches in signalling pathways, by coupling the activation of cell surface receptors to intracellular responses. Mutations in the G protein α-subunit (Gα) that accelerate guanosine diphosphate (GDP) dissociation cause hyperactivation of the downstream effector proteins, leading to oncogenesis. However, the structural mechanism of the accelerated GDP dissociation has remained unclear. Here, we use magnetic field-dependent nuclear magnetic resonance relaxation analyses to investigate the structural and dynamic properties of GDP bound Gα on a microsecond timescale. We show that Gα rapidly exchanges between a ground-state conformation, which tightly binds to GDP and an excited conformation with reduced GDP affinity. The oncogenic D150N mutation accelerates GDP dissociation by shifting the equilibrium towards the excited conformation.

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Heterotrimeric guanine-nucleotide-binding proteins (G proteins) serve as molecular switches in signalling pathways, by coupling the activation of G protein-coupled receptors (GPCRs) at the cell surface to intracellular responses. The signalling pathways, involving G proteins, play central roles in a wide variety of biological processes, including cellular division, immune responses and sensory functions. Heterotrimeric G proteins are composed of three subunits, an α-subunit (Gα), a β-subunit (Gβ) and a γ-subunit (Gγ). The latter two subunits are tightly associated and form a Gβγ dimer under physiological conditions. In the resting state, the G protein forms a heterotrimer, consisting of a guanosine diphosphate (GDP)-bound form of Gα (Gα-GDP) and Gβγ. Ligand binding to GPCRs induces conformational changes of Gα-GDP, promoting the dissociation of GDP. Guanosine triphosphate (GTP) spontaneously binds to Gα, resulting in the dissociation of the GDP-bound form of Gα (Gα-GTP) and Gβγ. Subsequently, both Gα-GTP and Gβγ bind to the effector proteins and regulate their functions, leading to cellular responses. The G protein returns to the resting state, following GTP hydrolysis and the re-association of Gα-GDP and Gβγ. The hydrolysis and the re-association of Gα-GDP are regulated by the binding of regulators of G protein signalling (RGS) to the G proteins, which controls both the length and strength of the effector regulations.

Based on primary sequence similarity, the Gα proteins can be classified into four major sub-families, i/o, s, q/11 and 12/13, and each couples with a different group of GPCRs and regulates different signalling pathways. The three-dimensional structures of Gα have a conserved protein fold, which is composed of a GTPase domain and a helical domain (Supplementary Fig. 1a). The GTPase domain consists of five helices (ξ1–ξ5) surrounding six β-sheets (β1–β6), and is homologous to the Ras-like small GTPase proteins. The helical domain consists of six helices (βA–βF), and is linked to the GTPase domain by two linker regions. The bound guanine nucleotide forms interactions with residues from both of the domains, and resides in a cleft between the two domains (Supplementary Fig. 1b,c). The GTPase domain contains three flexible loops, named switches 1, 2 and 3, where significant structural changes occur between the Gα-GTP and Gα-GDP structures. These regions are involved in binding to Gβγ, the effector proteins, and RGS.

Mutations in Gα have been found in various types of cancers, and the aberrant function of Gα is considered to be strongly associated with tumour gene expression. Genome sequencing studies have revealed that the mutations in Gα are frequently found in some tumour types, lacking B-Raf and N-Ras mutations, indicating that mutations in Gα potentially play a significant role in cancers. Among these oncopgenic mutations, some mutations, such as D151N (εε helix) and R243H (εε helix) in Gαo, are known to promote oncopgenesis by accelerating the GDP dissociation without the catalytic activity of the activated GPCR8–10. Since the intracellular GTP/GDP ratio is high (~10), the accelerated GDP dissociation leads to an increase in the fraction of active Gα-GTP, causing the hyperactivation of the effector proteins, which leads to oncopgenesis (Fig. 1a). Along with the sequencing analyses of cancer genomes, biochemical studies have also identified mutations with the accelerated GDP dissociation11–14. These mutated residues do not form direct interactions with the bound GDP, suggesting that the dissociation of GDP is facilitated in allosteric manners. In some cases, the crystal structures of the mutants have been solved; however, the structural differences in the GDP-binding modes have not clearly been observed. Therefore, the structural mechanism of the GDP dissociation and the means by which the oncopgenic mutations accelerate the GDP dissociation still remain unclear.

Here, we investigate the structural and dynamic properties of Gα-GDP with solution nuclear magnetic resonance (NMR) spectroscopy. Newly established NMR methods allow us to characterize chemical exchange processes on a microsecond timescale. We characterize the conformational exchange processes of wild-type Gα-GDP and its oncogenic D150N mutant, which allow us to explain the observed different GDP affinities of both proteins. Based on these results, we propose a dynamic regulatory mechanism for GDP dissociation.

Results

GDP dissociates faster in the D150N mutant. In this study, we focused on the D150N mutant of Gαo. The corresponding mutation has been found in Gαo, Gα16 and Gα2 in cancer. The dissociation rate of GDP is accelerated in the mutants, leading to a gain-of-function in cancer cells. We purified the wild-type and D150N mutant proteins and measured their GDP dissociation rate, using 3H-labelled GDP (3H-GDP) as a tracer. The dissociation rate constants of 3H-GDP were 0.0072 min−1 for the wild-type, and 0.14 min−1 for the D150N mutant at 20 °C. Thus, the GDP dissociation in the D150N mutant was ~20-fold faster than that in the wild type (Fig. 1b). Although the constitutive activation of the effector proteins by the D150N mutant has not been confirmed in cells, the Gαo mutant with the GDP dissociation rate sixfold faster than that of the wild type is reported to significantly enhance the activation of the effector proteins, and promote anchorage-independent growth in human mammary epithelial cells. Therefore, we conclude that the GDP dissociation rate in the D150N mutant is sufficiently high to promote the oncopgenic signalling.

The crystal structure of wild-type Gα-GDP (ref. 17) revealed that Asp150, located on the εε helix in the helical domain, forms no direct interactions with the bound GDP, therefore, the accelerated GDP dissociation seems to result from the structural differences in Gα-GDP induced by the mutation. To gain structural insights into the mechanism of the GDP dissociation, we conducted NMR analyses for the wild type and the D150N mutant. Because of the relatively large molecular weight of Gα-GDP (41 K), we adopted selective methyl-labelling strategies and applied methyl-TROSY techniques. We prepared a selectively labelled [u-13H, Ile61, Leu62, Val72-[13C6]]-Gα-GDP sample, and compared the 1H-13C heteronuclear multiple quantum (MQ) coherence (HMQC) spectra (Fig. 1c and Supplementary Fig. 2). Remarkably, the chemical shift differences between the wild type and the D150N mutant were quite small, and the weighted average chemical shift differences were <0.1 p.p.m. for all methyl groups, suggesting that the overall structure is well preserved in the mutant. Chemical shift differences larger than 0.03 p.p.m. were observed for Leu148, Val174, Val223 and Leu234, which did not form direct contacts with the bound GDP, suggesting that the interactions with the bound GDP were not significantly perturbed in the D150N mutant (Fig. 1e).

Numerous studies have shown that ligand dissociation occurs from a low-affinity structure, which is only transiently formed and is exchanging with the ground-state structure. In support of this idea, an increase in conformational flexibility during nucleotide exchange has been noted in previous structural studies of Gα (refs. 14,22). To determine whether such conformational exchange processes exist and contribute to the GDP dissociation process in Gα-GDP, we conducted MQ Carr–Purcell–Meiboom–Gill relaxation dispersion (MQ CPMG RD) experiments, to characterize the chemical exchange processes on millisecond to microsecond timescales (Fig. 1d,e). In the wild type, small but significant differences in the effective relaxation rates (Reff)
In cells expressing the Gα wild type.

The experiments were performed at 14.1 T tesla (600 MHz 1H frequency). The error bars represent the experimental errors calculated using the equation (16).

The 13CH2H2 labelling results in lower sensitivities (about 0.2–0.4) compared with the 1H labelling, mainly due to the reduced initial magnetization 26. Further, to detect and characterize the chemical exchange processes on a accessible timescales are often limited to exchange rates of 102–103 s−1, mainly due to the upper limit of the frequencies of the CPMG pulse trains. The faster exchange processes, with exchange rates on the order of 103–104 s−1, can be characterized by spin-lock-based R1rho dispersion experiments 24. However, when observing methyl groups, the R1rho dispersion experiments require 13CH2H2 labelled samples, in order to avoid artefacts derived from intra-methyl dipolar cross-correlation 25. The 13CH2H2 labelling results in lower sensitivities (about threefold), mainly due to the reduced initial magnetization 26. Therefore, the applications of the R1rho dispersion experiments have been limited to small- or medium-sized proteins, so far 25,27, and a new strategy is needed for characterizing the microsecond-order conformational exchange processes in high molecular weight proteins, such as Gα-GDP.

To overcome these difficulties, we developed an NMR method to detect and characterize the chemical exchange processes on a microsecond timescale (an exchange rate of 103–104 s−1). We also conducted the MQ CPMG RD experiments with the wild type (black) and the D150N mutant (red). The methyl groups with chemical shift differences larger than 0.03 p.p.m. are shown labelled. (e) Mapping of the methyl groups with chemical shift differences in the D150N mutant on the structure of Gα-GDP (PDB ID: 1GDD) 17. Methyl groups with chemical shift differences larger than 0.03 p.p.m. are coloured orange. Methyl groups with no data are coloured grey. The methyl groups for which MQ CPMG RD results are presented (Leu38, Leu39, Ile85 and Ile253) are also shown with labels.

Figure 1 | Functional and structural analyses of the wild-type and mutant Gα-GDP. (a) Proposed mechanism for oncogenesis induced by the Gα mutant 9. In cells expressing the Gα mutant that shows accelerated GDP dissociation, the GDP-GTP exchange reaction is facilitated without the activated GPCR, and the fraction of Gα-GTP is increased. The increased Gα-GTP causes hyperactivation of the effector proteins, leading to oncogenesis. (b) 3H-GDP dissociation assays of the wild-type Gα and the D150N mutant. The experiments were performed at 20 °C. Each point reflects mean ± s.e. of three independent experiments. (c) Overlay of the 1H-13C HMQC spectra of the wild type (black) and the D150N mutant (red). The methyl groups with chemical shift differences larger than 0.03 p.p.m. are shown labelled. (d) Results of the MQ CPMG RD experiments with the wild type (black) and the D150N mutant (red). The experiments were performed at 14.1 Tesla (600 MHz 1H frequency). The error bars represent the experimental errors calculated using the equation (16).

These results demonstrated that conformational exchange processes exist in Gα-GDP, and the exchange parameters are different between the wild type and the D150N mutant, suggesting that the exchange processes are closely related to the GDP dissociation. However, the differences in the R2,eff rates were small and detected in only a few methyl groups, thus hampering the quantitative and comprehensive analyses of the conformational exchange processes in Gα-GDP.

MQ relaxation analyses of Gα-GDP. Although the MQ CPMG RD experiments are beneficial for characterizing the chemical exchange processes in high molecular weight proteins, the accessible timescales are often limited to exchange rates of 102–103 s−1, mainly due to the upper limit of the frequencies of the CPMG pulse trains. The faster exchange processes, with exchange rates on the order of 103–104 s−1, can be characterized by spin-lock-based R1rho dispersion experiments 24. However, when observing methyl groups, the R1rho dispersion experiments require 13CH2H2 labelled samples, in order to avoid artefacts derived from intra-methyl dipolar cross-correlation 25. The 13CH2H2 labelling results in lower sensitivities (about threefold), mainly due to the reduced initial magnetization 26. Therefore, the applications of the R1rho dispersion experiments have been limited to small- or medium-sized proteins, so far 25,27, and a new strategy is needed for characterizing the microsecond-order conformational exchange processes in high molecular weight proteins, such as Gα-GDP.

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microsecond timescale from the side-chain methyl $^{1}$H-$^{13}$C MQ relaxation rates, by utilizing the static magnetic field dependence. Assuming a two-state model (states A and B), chemical exchange processes, the exchange contributions in the $^{1}$H-$^{13}$C MQ relaxation rates, $R_{M/Q(ex)}$ and $AR_{M/Q(ex)}$, can be expressed by equations (1) and (2), using the exchange rate, $k_{ex}$, the populations of the two states, $p_{A}$ and $p_{B}$, and the $^{1}$H and $^{13}$C chemical shift differences given in p.p.m., $\Delta \omega_{C}$ and $\Delta \omega_{H}$ (refs 31,32).

$$R_{M/Q(ex)} = \frac{\gamma_{C}^{2} \Delta \omega_{C}^{2} + \gamma_{H}^{2} \Delta \omega_{H}^{2}}{k_{ex}} p_{PB} B_{0}^{2}$$

$$AR_{M/Q(ex)} = \frac{4 \gamma_{C}^{2} \Delta \omega_{C}^{2} \gamma_{H}^{2} \Delta \omega_{H}^{2}}{k_{ex}} p_{PB} p_{A} B_{0}^{2}$$

The $R_{M/Q(ex)}$ and $AR_{M/Q(ex)}$ rates can be extracted, using (i) the $R_{M/Q}$ and $AR_{M/Q}$ rates measured at different static magnetic fields, (ii) the chemical shift anisotropy (CSA) values, $\Delta \sigma_{C}$ and $\Delta \sigma_{H}$ and (iii) the product of the order parameter and the rotational correlation time, $S_{ax}^{2} T_{2}^{C}$, as summarized in Fig. 2a. We verified that the $R_{M/Q(ex)}$ and $AR_{M/Q(ex)}$ rates are highly sensitive to the chemical exchange processes on a microsecond timescale, and that accurate $R_{M/Q(ex)}$ and $AR_{M/Q(ex)}$ rates can be obtained by the magnetic field-dependent MQ relaxation analyses in high molecular weight proteins (Supplementary Figs 3–6).

To examine the conformational exchange processes in Gz-GDP, we applied the magnetic field-dependent MQ relaxation analyses to Gz-GDP. Figure 2b shows the plots of the $R_{M/Q}$ rates of Leu38, Leu39, Ile85 and Ile253, as a function of $B_{0}$. Significantly large magnetic field-dependent changes were observed for Leu38, Leu39 and Ile253, reflecting the $R_{M/Q(ex)}$ rates over 20 s$^{-1}$ at 14.1 Tesla (600 MHz $^{1}$H frequency). These results were in contrast to the results from the MQ CPMG RD analyses, where the observed differences in the $R_{M/Q}$ rates were as large as 5.0 s$^{-1}$, demonstrating the robustness of the method. The plots of the $R_{M/Q(ex)}$ and $AR_{M/Q(ex)}$ rates at 14.1 Tesla are shown in Fig. 2c, and the methyl groups with significant $R_{M/Q(ex)}$ and $AR_{M/Q(ex)}$ rates were mapped on the structure of Gz-GDP (Fig. 2d). These methyl groups were located on the $^{1}$H-$^{13}$C HMQC spectra of a $^{1}$H-$^{15}$N, Ala$^{15}$-[13CH$_{3}$]-Gz-GDP in the presence (red) and absence (black) of 20 mM Mg$^{2+}$. Upon the addition of Mg$^{2+}$, some NMR signals exhibited chemical shift changes. The apparent dissociation constant of Mg$^{2+}$ was calculated to be 3–5 mM, from the intensity ratio of the two signals (Fig. 3c). The methyl groups with significant chemical shift changes over 0.02 p.p.m. were Ile49 (z1-helix), Ile222 (b4-strand), Ile253 (z3-helix) and Ile264 (b5-strand). These residues were not clustered in the vicinity of the Mg$^{2+}$-binding site identified in the crystal structure (PDB ID: 1BOF) (ref. 34), suggesting that the Mg$^{2+}$ binding allosterically induces conformational changes in Gz-GDP (Fig. 3d). In the NMR analyses conducted by Goricanec et al. 33, the experiments were performed in the presence of 5 mM Mg$^{2+}$, and the methyl groups with significant chemical shift changes were observed in the $^{1}$H-$^{15}$N TROSY spectra. Indeed, the two Phe191 amide signals are very similar to the splitting of the $^{1}$H-$^{15}$N amide signal observed by Goricanec et al. (Fig. 7 in ref. 33).

Furthermore, we observed a chemical exchange process on a millisecond timescale in the presence of 5 mM Mg$^{2+}$. Figure 3e shows the result of the $^{13}$C SQ CPMG dispersion experiments of Ile49 and Ile222 in the presence and absence of 5 mM Mg$^{2+}$. The results clearly demonstrate that significant changes in the $R_{M/Q}$ rates are observed only in the presence of 5 mM Mg$^{2+}$. The fitted $\Delta \omega_{C}$ and $p_{B}$ values were calculated to be 0.44 p.p.m., 96 s$^{-1}$ and 0.12 for Ile49 and 0.26 p.p.m., 48 s$^{-1}$ and 0.22 for Ile222, respectively. Since the sizes of the $\Delta \omega_{C}$, $\Delta \omega_{H}$ and $p_{B}$ values are not significantly different between the two signals observed in the presence of Mg$^{2+}$, we assume that the chemical exchange process observed in the presence of 5 mM Mg$^{2+}$ reflects the exchange process between the two conformations, induced by Mg$^{2+}$ binding. We also confirmed that the magnetic field-dependent changes in the $R_{M/Q}$ and $AR_{M/Q}$ rates were still observed in the presence of Mg$^{2+}$. These results support our proposal that the millisecond exchange processes observed in the presence of Mg$^{2+}$ are different exchange processes from those observed in the magnetic field-dependent MQ relaxation analyses, and these distinct exchange processes are probably related to the different functions of Gz.

Thus, we conclude that the differences in the results of the CPMG RD experiments are mainly due to different Mg$^{2+}$ concentrations, and that the existence of the chemical exchange on a millisecond timescale is consistent with the results of the CPMG RD experiments by Goricanec et al. 33, which were performed in the presence of 5 mM Mg$^{2+}$. However, it should be noted that we did not detect significant changes in the $R_{M/Q}$ rates in the methyl groups from the helical domain (Leu159) and the C-terminal region (Val342) in the presence of 5 mM Mg$^{2+}$, although significant changes in the $R_{M/Q}$ rates were observed for these methyl groups in the SQ CPMG RD analyses by Goricanec.

The effect of Mg$^{2+}$ on Gz-GDP. The results from the magnetic field-dependent MQ relaxation analyses revealed that Gz-GDP undergoes a conformational exchange process, and successfully identified the regions where significant structural rearrangements occur. Together with the results of the MQ CPMG RD analyses, the timescales of the exchange processes are faster than those amenable to the CPMG RD approaches, and estimated to be on a microsecond order (the exchange rates of $10^{3}$–$10^{4}$ s$^{-1}$). Recently, Goricanec et al. 33 demonstrated that Gz$_{31}$α31-GDP exists in chemical exchange processes on a millisecond timescale ($\sim$890 s$^{-1}$), as revealed by the splitting of $^{1}$H-$^{15}$N amide resonances and the single quantum (SQ) CPMG RD analyses. These results are apparently inconsistent with our results, which show that Gz$_{31}$α31-GDP gives a single set of $^{1}$H-$^{13}$C methyl resonances and exists in chemical exchange processes on a microsecond timescale ($\sim$10$^{3}$–$10^{4}$ s$^{-1}$) which are hardly detected by the CPMG RD analyses. We suppose that these differences are attributable to the different Mg$^{2+}$ concentrations in the NMR buffers.
Figure 2 | Magnetic field-dependent MQ relaxation analyses of the wild-type Gx-GDP. (a) Schematic representation of the magnetic field-dependent MQ relaxation analyses. (b) Plots of the $R_{MQ}$ rates of Leu38, Leu39, Ile85 and Ile253 against the square of the static magnetic fields. The obtained $R_{MQ}$ rates at 14.1 Tesla (600 MHz $^1$H frequency) are shown. The error bars represent s.d. of fitting errors, estimated from Monte Carlo simulations using uncertainties in peak intensities. (c) Plots of the $R_{MQ}$,ex (top) and $\Delta R_{MQ}$,ex (bottom) rates at 14.1 Tesla. The error bars represent s.d. of fitting errors, estimated from Monte Carlo simulations using uncertainties in peak intensities. The methyl groups with $R_{MQ}$,ex or $\Delta R_{MQ}$,ex rates larger than 10 s$^{-1}$ are coloured magenta, and those within the 5-10 s$^{-1}$ range are coloured pink. The small negative values of the $R_{MQ}$,ex rates are due to the overestimations to the CSA contributions to the MQ relaxation rates, originating from systematic errors in the measurements. We suppose that the relatively large negative value of the $R_{MQ}$,ex rate of Val332 is due to its low signal-to-noise ratio and its very large dipolar contribution to the MQ relaxation rate (44 s$^{-1}$), which hampers the reliable measurement of the $^1$H-$^1$C cross-correlated relaxation rate (1.9 s$^{-1}$ on average). (d) Mapping of the methyl groups with significant $R_{MQ}$,ex or $\Delta R_{MQ}$,ex rates on the structure of Gx-GDP (PDB ID: 1GDD)$^{17}$. The methyl groups with significant $R_{MQ}$,ex or $\Delta R_{MQ}$,ex rates are coloured in the same manner as in c. Methyl groups with no data are coloured grey.
Figure 3 | Effects of Mg\(^{2+}\) binding on G\(\alpha\)-GDP. (a) Overlay of the \(^1\)H-\(^{15}\)N TROSY spectra of \((u-^2\)H, \(^{15}\)N, Ala\(\beta\), lle\(\delta\1-[13CH₃]\)\) G\(\alpha\)-GDP in the presence (red) and absence (black) of 20 mM Mg\(^{2+}\). The Mg\(^{2+}\) concentration-dependence of the Phe191 signal is shown below. (b) Overlay of the \(^1\)H-\(^{13}\)C HMQC spectra of \((u-^2\)H, \(^{15}\)N, Ala\(\beta\), lle\(\delta\1-[13CH₃]\)\) G\(\alpha\)-GDP in the presence (red) and absence (black) of 20 mM Mg\(^{2+}\). The Mg\(^{2+}\) concentration-dependence of the Ile49 signal is shown below. (c) The intensity ratios of the two signals observed for Phe191 and Ile49 were plotted against the Mg\(^{2+}\) concentration. The apparent dissociation constants calculated from the Phe191 and Ile49 signals were 3.1 and 3.0 mM, respectively. (d) The Ala and lle methyl groups with significant chemical shift differences are mapped on the crystal structure of G\(\alpha\)-GDP bound to Mg\(^{2+}\) (PDB ID: 1BOF)\(^3\)\(^4\). The methyl groups with chemical shift differences larger than 0.02 p.p.m. are coloured red, and those within the 0.01–0.02 p.p.m. range are coloured pink. (e) \(^{13}\)C SQ CPMG dispersion experiments of Ile49 and Ile222. The results in the presence of 5 mM Mg\(^{2+}\) are coloured red, and those in the absence of Mg\(^{2+}\) are coloured black. The error bars represent the experimental errors calculated using the equation (16). The fitted parameters are summarized below. The measurements were performed at 20 °C with a Bruker Avance 600 spectrometer. The \(^{13}\)C HMQC spectra of the corresponding residues are also shown.
We suppose these variations are originated from the differences in the construct (G2a3,Δ31 and G2a3), the buffer conditions and the temperature, and further experimental studies are required to clarify these points.

Since the intracellular free Mg$^{2+}$ concentration is reportedly within the range of 0.2–1.2 mM (refs 35,36), the majority of G$\tau $-GDP ( > 70 %) is estimated to be in the Mg$^{2+}$-bound state under physiological conditions. Therefore, we emphasize that our results have been obtained under physiologically relevant conditions. In addition, we confirm that the absence of Mg$^{2+}$ does not preclude the analysis of the GDP dissociation process, because Mg$^{2+}$ reportedly does not affect the rate of GDP dissociation from G$\tau $ (ref. 37).

**MQ relaxation analyses of the D150N mutant.** We applied the magnetic field-dependent MQ relaxation analyses to the D150N mutant, which exhibited the faster dissociation of GDP, and compared the results with those from the wild type. Figure 4a shows the plots of the $R_{\text{MQ,ex}}$ rates of the D150N mutant at 14.1 Tesla (600 MHz $^1$H frequency). While significantly large $R_{\text{MQ,ex}}$ rates were observed in the same methyl groups as the wild type, the $R_{\text{MQ,ex}}$ rates in Leu38, Leu39 and Val201 were larger than those of the wild type. These methyl groups are clustered on the $\beta$-1-strand (Leu38, Leu39) and the adjacent $\beta$-3-strand (Val201; Fig. 4b), indicating that the conformational exchange processes in the $\beta$1 strand are facilitated in the D150N mutant. In the crystal structure of G$\tau $-GDP (ref. 17), the side-chain of Asp150 forms a hydrogen bond network with the Lys270 ($\beta$5-strand) side-chain and the Glu43 (P-loop) main-chain carbonyl group. Therefore, the substitution of Asp to Asn could cause the disruption of the network, leading to the altered dynamics in the P-loop and adjacent $\beta$-1-strand.

To further characterize the exchange process, we analysed the peak positions of Leu38, Leu39 and Val201. In the D150N mutant, the peak positions were slightly different from those in the wild type (Fig. 4c). If the observed differences in the peak positions reflect the shift in the conformational equilibrium in the D150N mutant, then the corresponding $^1$H-$^1$C MQ chemical shift differences, that is $(\gamma^1_\text{H} \Delta \Delta^1_\text{C} + \gamma^1_\text{H} \Delta \Delta^1_\text{P} \text{ equation (1)})$ and $\gamma^4_\text{C} \Delta \Delta^4_\text{CH} (\Delta \Delta^4_\text{P} \text{ equation (2)})$, should be correlated to the $R_{\text{MQ,ex}}$ and $\Delta R_{\text{MQ,ex}}$ rates, respectively. Indeed, the MQ chemical shift differences calculated from the differences in the peak positions were highly correlated to the $R_{\text{MQ,ex}}$ and $\Delta R_{\text{MQ,ex}}$ rates ($R = 0.88$), strongly suggesting that the increase in the $R_{\text{MQ,ex}}$ rates reflects the shift in the conformational equilibrium, and that the minor state population is increased in the D150N mutant (Fig. 4d).

**The effect of the binding of the GoLoco14 peptide.** To further investigate the relationship between the GDP dissociation and the conformational exchange processes in G$\tau $-GDP, we subsequently applied the method to G$\tau $-GDP bound to the GoLoco14 peptide, the G$\tau $-binding motif of the RGS14 protein. The GoLoco14 peptide selectively binds to the G$\tau $-GDP state in the signalling cascade, and inhibits the GDP dissociation. Therefore, we investigated the conformational exchange processes in G$\tau $-GDP bound to the GoLoco14 peptide, where the GDP dissociation is suppressed.

We prepared a peptide containing the GoLoco14 sequence (GoLoco14), and confirmed that the $^2$H-GDP dissociation was inhibited in its presence (Fig. 5a). We measured the IC50 value of GoLoco14, from the concentration dependency of the inhibition of the $^2$H-GDP dissociation. The IC50 of GoLoco14 was calculated to be 620 ± 150 nM, consistent with the previous report of IC50 values in the sub-micromolar range (Fig. 5b). The binding of GoLoco14 was also confirmed from the chemical shift changes observed in the $^1$H-$^1$C HMQC spectra upon the addition of GoLoco14 (Supplementary Fig. 8).

We applied the magnetic field-dependent MQ relaxation analyses to GoLoco14-bound G$\tau $-GDP (GoLoco14-G$\tau $), and compared the results with those obtained in the absence of GoLoco14. Figure 5c shows the plots of the $R_{\text{MQ,ex}}$ rates of GoLoco14-G$\tau $ at 14.1 Tesla (600 MHz $^1$H frequency), in comparison with the results obtained in the absence of GoLoco14. Strikingly, the $R_{\text{MQ,ex}}$ rates in GoLoco14-G$\tau $ were smaller than those in the absence of GoLoco14, for the majority of the methyl groups. The methyl groups that showed the reduced $R_{\text{MQ,ex}}$ rates were Leu38, Leu39, Ile55, Val174, Ile221 and Leu234. These methyl groups are located on the $\beta$1-strand (Leu38, Leu39), $\alpha$1-helix (Ile55), $\alpha$F-helix (Val174) and $\beta$4–$\gamma$3 loop (Leu234), indicating that the conformational exchange processes in these regions are suppressed by the binding of GoLoco14 (Fig. 5d).

Notably, the conformational exchange processes in the $\beta$1 strand (Leu38, Leu39) were suppressed by the binding of GoLoco14, in contrast to the results obtained with the D150N mutant, in which the conformational exchange processes in the $\beta$1-strand were facilitated. These results strongly suggest that the conformational exchange processes in the $\beta$1-strand are closely related to the GDP dissociation.

**Discussion**

Here, we developed magnetic field-dependent MQ relaxation analyses for characterizing the chemical exchange processes on a microsecond timescale in high molecular weight proteins. The results from G$\tau $-GDP revealed that the method is particularly beneficial for characterizing the chemical exchange processes on a microsecond timescale, which are difficult to detect by the conventional CPMG RD experiments. Moreover, the method utilizes the measurements of the $^1$H-$^1$C MQ relaxation rates and benefits from the methyl-TROSY principle, enabling the analyses of conformational equilibrium processes in high molecular weight proteins. Although the extraction of the exchange parameters ($k_{\text{ex}}Pb$ and $\Delta \tau$) is usually difficult to achieve solely from the observed $R_{\text{MQ,ex}}$ and $\Delta R_{\text{MQ,ex}}$ rates, the high sensitivities to the existence of chemical exchange processes and the linearity in the MQ chemical shift differences would greatly facilitate the characterization of the conformational exchange phenomena. It is becoming increasingly apparent that conformational exchange processes on a microsecond timescale are closely related to various kinds of protein functions, such as molecular recognition, ligand binding, signal transduction and receptor activation. So far, the analyses of the exchange processes on a microsecond timescale have been restricted to small- and medium-sized proteins, due to the limited sensitivities of the existing methods. Applications of the magnetic field-dependent MQ relaxation analyses will accelerate the elucidations of the functional dynamics of biologically significant proteins with high molecular weights, such as membrane proteins and functional protein complexes.

We applied the magnetic field-dependent MQ relaxation analyses to G$\tau $-GDP, and identified the regions that exist in a conformational exchange process. Our analyses revealed that the conformational exchange processes observed in the $\beta$1-strand are facilitated in the D150N mutant, which exhibits the accelerated GDP dissociation, while the exchange processes are suppressed by the binding of GoLoco14, which inhibits the GDP dissociation. These results suggest that the conformational exchange processes in the $\beta$1-strand play a fundamental role in the GDP dissociation.

As shown from the comparisons between the $R_{\text{MQ,ex}}$ and $\Delta R_{\text{MQ,ex}}$ rates and the differences in the MQ chemical shifts,
the fraction of the minor state in the equilibrium seems to be increased in the D150N mutant, as compared with the wild-type. Taken together, we propose the regulatory mechanism of the GDP dissociation driven by the conformational exchange, as summarized in Fig. 6. The β1-strand region of Gα-GDP exists in a conformational equilibrium, and the minor conformation in the equilibrium has significantly reduced affinity to GDP. The ground-state conformation is assumed to be stabilized by the hydrogen bond network formed by Asp150, Lys270 and Glu43, which anchor the β1-strand and the P-loop to the bound GDP as observed in the crystal structure (Figs 4b and 6b). In the D150N mutant, the ground-state conformation is destabilized by weakening of the hydrogen bond network, due to the loss of the negative charge on the side chain, and the conformational equilibrium shifts towards the minor state, leading to the accelerated dissociation of GDP (Fig. 6c). However, the binding of GoLoco14 suppresses the conformational exchange processes in the β1-strand and stabilizes the ground-state conformation, which tightly binds to GDP, leading to the inhibition of the GDP dissociation (Fig. 6a). The analyses of the 1H line widths of the

Figure 4 | Magnetic field-dependent MQ relaxation analyses of the D150N mutant. (a) Plots of the $R_{MQ,ex}$ rates of the wild type (light grey) and those of the D150N mutant (red) at 14.1 Tesla (600 MHz $^1$H frequency). The error bars represent s.d. of fitting errors, estimated from Monte Carlo simulations using uncertainties in peak intensities. The methyl groups with increased $R_{MQ,ex}$ rates in the D150N mutant are highlighted. (b) Expanded view of the Leu38, Leu39 and Val201 methyl groups in the crystal structure of Gα-GDP (PDB ID: 1GDD)17. The methyl carbons are shown as red spheres. Lys46, Lys270 and Asp150 are shown as sticks. (c) The overlay of the $^1$H-$^1$C HMQC spectra of Leu38, Leu39 and Val201. The spectra of the wild type are coloured black, and those of the D150N mutant are coloured red. (d) Linear correlation plot of the $R_{MQ,ex}$ (triangles) and $\Delta R_{MQ,ex}$ rates (circles) against the MQ chemical shift differences between the wild type and the D150N mutant for Leu38, Leu39 and Val201. The error bars represent s.d. of fitting errors, estimated from Monte Carlo simulations using uncertainties in peak intensities.
amide resonances support that the conformational equilibrium processes exist in the P-loop region, which directly interacts with the bound GDP through amide–phosphate interactions. Therefore, the reduced affinity to GDP in the minor state conformation seems to be mainly attributed to the altered interaction mode of the P-loop with the GDP phosphates. Our proposed scheme indicates that the dissociation of GDP is driven by the conformational dynamics of Gα, and may be generally applied to the functional mechanisms of other Gα mutants and the ligands that modulate Gα functions.

Rearrangements in the hydrogen bond network formed by Asp150 have not been observed in the crystal structures with the reduced affinity to GDP. Therefore, it has been difficult to elucidate the structural mechanism of the accelerated GDP dissociation in the D150N mutant by simply comparing the crystal structures. The crystal structures represent the ground-state structures tightly bound to GDP, and provide the limited insights into the excited state structures, which is only transiently formed and play critical roles in the GDP dissociation. In contrast, the NMR results reveal the existence of conformational

**Figure 5 | Effects of the binding of GoLoco14.** (a) 3H-GDP dissociation assays of Gα-GDP in the presence (cyan) and absence (black) of 10 μM GoLoco14. The experiments were performed at 30 °C. Each point reflects mean ± s.e.m. of three independent experiments. (b) Plot of the percentage inhibition of the 3H-GDP dissociation against the concentration of GoLoco14. The percentage inhibition was calculated from the amount of bound 3H-GDP after a 10 min incubation in the presence of various concentrations of GoLoco14 (100 nM to 10 μM). The calculated IC50 value was 620 ± 150 nM. Each point reflects mean ± s.e. of three independent experiments. (c) Plots of the RMQ,ex rates in the presence (cyan) and absence (light grey) of GoLoco14 at 14.1 Tesla (600 MHz 1H frequency). The error bars represent s.d. of fitting errors, estimated from Monte Carlo simulations using uncertainties in peak intensities. The methyl groups with reduced RMQ,ex rates in the presence of GoLoco14 are highlighted. We suppose that the increase in the RMQ,ex rates of Val201 is mainly due to the change in the Δν value, induced by the binding of GoLoco14 (~ 0.4 p.p.m., Supplementary Fig. 8b). (d) Mapping of the methyl groups with reduced RMQ,ex rates in the presence of GoLoco14 on the structure of GoLoco14-Gα-GDP (PDB ID: 1KJY). The methyl groups with reduced RMQ,ex rates in the presence of GoLoco14 are coloured cyan. Methyl groups with no data are coloured grey.
equilibrium processes, and provide structural insights into the excited state structure from the measurements of the $R_{MQ,ex}$ and $\Delta R_{MQ,ex}$ contributions. In this point of view, our NMR study advances the mechanistic understanding in the functions of the G proteins, and precisely complements the crystallographic studies so far.

Our NMR results also indicate that the conformational exchange processes exist in the switch regions. Studies of the small GTPase Ras have revealed that the GTP-bound Ras is exchanging between different conformational states, called state 1 and state 2, which show structural differences in switch 1 and switch 2, and that the transitions between these two states are closely related to the guanine nucleotide association/dissociation kinetics$^{47-49}$. Given the high structural homology between the Gα-GTPase domain and Ras, the conformational exchange processes observed in Gα might correspond to the state 1 to state 2 transition in Ras.

Although our study has focused on the GDP dissociation from Gα, in the absence of the catalytic activity of an activated GPCR, the modulation of the conformational dynamics in the β1 strand is also suggested to occur in the GPCR-catalysed GDP dissociation. Peptide amide hydrogen-deuterium exchange mass spectroscopy analyses, using activated β2-adrenergic receptor (β2AR) and Gα proteins, identified the β1-strand as the region where structural rearrangements occur upon GPCR activation$^{50}$. Since the structural differences observed in the β1-strand were very small between the crystal structures of Gα-GDP and the Gα-GTP complex$^{51}$, the role of the structural rearrangements in the β1-strand has remained unclear. Considering our NMR results, the activated GPCR may promote the spontaneous GDP release, by modulating the conformational equilibrium within Gα and stabilizing the minor state conformation with the reduced GDP affinity.

In summary, we established the magnetic field-dependent NMR relaxation analyses, and revealed that Gα exchanges between a ground-state conformation, which tightly binds to GDP, and an excited conformation with reduced affinity to GDP. The oncogenic mutation and the binding of GoLoco14 regulate the dissociation of GDP by modulating the conformational equilibrium processes within Gα and stabilizing the minor state conformation with the reduced GDP affinity.

In conclusion, our study provides the structural insights into the activation mechanism of G proteins, and demonstrates the importance of the conformational dynamics of Gα in regulating G protein signalling.

**Methods**

**Protein expression and purification.** The human Gα$_i$ (residues 1–354) protein, including an N-terminal His10-tag and an HRV-3C protease recognition site, was expressed in *Escherichia coli* BL21 (DE3) cells (Agilent Technologies). For the selective $^{13}$C$_2$-labelling of methyl groups, *E. coli* cells were grown in deuterated

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**Figure 6 | Schematic representation of the conformational equilibrium in Gα-GDP and the regulation of the GDP dissociation.** (a) The binding of GoLoco14 suppresses the conformational exchange processes and stabilizes the ground-state conformation that tightly binds to GDP, leading to the inhibition of the GDP dissociation. (b) The β1-strand and P-loop regions exist in an equilibrium between conformations with high and low affinity to GDP. The low-affinity conformation promotes the GDP dissociation. (c) The conformational equilibrium shifts towards the low-affinity conformation in the D150N mutant, causing the accelerated GDP dissociation.
M9 media and 300 mg l⁻¹ of [methyl-13C, 3-H2]-α-ketobutyric acid (for Lle51) (CIL) and 300 mg l⁻¹ of [2-methyl-13C, 4-2H3]-acetolactate (for Leu2 and Val2) (NMR Biochem) were both added 1 h before the induction. The cells were purified by affinity chromatography using His 10-tag (refs 54, 55). Briefly, the cell suspension was first cleared by centrifugation, and the cell-free supernatant was loaded onto a His-Select resin column (Amersham Pharmacia Biotech). The column was then washed extensively with a wash buffer (50 mM HEPES pH 7.0, 0.5 mM GDP and 5 mM DTT in D2O). The D150N mutant was constructed with a QuickChange Site-directed Mutagenesis Kit (Agilent Technologies).

The GoLoCo14 peptide (residues 496-531 of rat RGSI4), including an N-terminal glutathione S-transferase (GST)-tag, was expressed in E. coli C41 (DE3) cells (Lucigen). The peptide was purified with Glutathione sepharose (GE). Under these assumptions, the CSA and exchange contributions, respectively. The magnetic field-dependency has been utilized for MQ relaxation analyses (T.D. Goddard and D.G. Kneller, Sparky 3, University of California, San Francisco, CA, USA).

Establishment of MQ relaxation analyses. To evaluate the chemical exchange contributions to the MQ relaxation rates, we exploited the fact that the exchange contributions increase linearly with the square of the static magnetic field strength in a fast exchange regime. The magnetic field-dependency has been utilized for characterizing exchange processes, by observing the backbone 1H-15N resonances28-30. In the side-chain methyl groups, the 1H-13C MQ relaxation rates RMQ (equation (3)) and the differential MQ relaxation rates ΔRMQ (equation (4)) can be expressed as the sum of the dipolar contributions (with the subscript DD), the CSA contributions (with the subscript CSA), and the exchange contributions (with the subscript ex) (equations (3) and (4)):

\[ R_{MQ} = R_{MQ,DD} + R_{MQ,CSA} + R_{MQ,ex} \]  

\[ ΔR_{MQ} = ΔR_{MQ,DD} + ΔR_{MQ,CSA} + ΔR_{MQ,ex} \]

Here, we assume a slow tumbling limit (\( 10^2 > 3 \)), where \( 10^2 \) represents Larmor frequency and \( 3 \) represents rotational correlation time) and a fast exchange regime (exchange rates, \( 3 > 10^2 \) chemical shift differences, \( 30 \)). In the case of proteins with molecular weights larger than 20 kDa (corresponding to the correlation time of \( 10^{-2} \)), the value of \( 10^2 \) is estimated to be larger than \( 10^2 \). In addition, the MQ chemical shift differences in 1H-13C methyl groups are usually smaller than 500 Hz at 14.1 Tesla, as judged from the case of T4 lysozyme mutant, which is below the magnetic field of 10 T. Thus, the assumption of a slow tumbling limit and a fast exchange regime usually hold for the microsecond exchange processes in high molecular weight proteins. Under these assumptions, the CSA and exchange contributions are both proportional to the square of the static magnetic field strength, \( B_0 \).

The CSA contributions, \( R_{MQ,CSA} \) and \( ΔR_{MQ,CSA} \), can be expressed by equations (5) and (6), using the gyromagnetic ratios, \( γ_C \) and \( γ_H \), the CSA values, \( ΔC \) and \( ΔH \), the methyl threefold axis order parameter, \( S_{ax} \), the rotational correlation time, \( τ_C \), and the angle between the principal axis of the CSA tensor, \( θ_{CH} \), and \( θ_{CCH} \):

\[ R_{MQ,CSA} = \frac{4\left(γ_C^2ΔC^2 + γ_H^2ΔH^2\right)}{45} \cdot \frac{15}{3} \cdot B_0^3 \]

\[ ΔR_{MQ,CSA} = 16\left(γ_C^2ΔC^2\right)\frac{4}{45} \cdot \frac{15}{3} \cdot \cos3θ_{CH} \cdot \left(1 + \frac{1}{3}\right) B_0^3 \]

Assuming a two-state (states A and B) chemical exchange process, the exchange contributions, \( R_{MQ,ex} \) and \( ΔR_{MQ,ex} \), can be expressed by equations (1) and (2) in the main text10,12. The \( R_{MQ,ex} \) rates are proportional to the sum of \( γ_C^2ΔC^2 \) and \( γ_H^2ΔH^2 \) while the \( ΔR_{MQ,ex} \) rates are proportional to the product of \( γ_C^2ΔC^2 \) and \( γ_H^2ΔH^2 \). Therefore, the analysis of both the \( R_{MQ,ex} \) and \( ΔR_{MQ,ex} \) rates would provide detailed information about the exchange processes, such as the relative signs of \( ΔC \) and \( ΔH \).

On the basis of the equations (1)-(6), the chemical exchange contributions can be extracted using (i) the \( R_{MQ} \) and \( ΔR_{MQ} \) rates measured at different static magnetic fields, (ii) the CSA values, \( ΔC \) and \( ΔH \), and (iii) the products of the order parameter and the rotational correlation time, \( S_{ax}τ_C \), as summarized in Fig. 2a.

The \( R_{MQ} \) and \( ΔR_{MQ} \) rates are measured by Hahn-echo type relaxation measurements, established by Gill and Palmer23. During the Hahn-echo relaxation period \( T (n=2,3) \), double quantum (DQ) or zero quantum (ZQ) coherences are generated and the relaxation rates were extracted by fitting the exponential decay function (equation (7)). The relaxation rate measured during the relaxation period, \( T \), represents the relaxation rates (\( R_{MQ} \) or \( ΔR_{MQ} \))

\[ \frac{1}{T} = 1 + \exp\left(-\frac{R_{MQ}}{R_{sol}}\right) \]  

\[ ΔR_{MQ} = R_{sol} - R_{sol} \]

In current applications, the \( R_{MQ} \) and \( ΔR_{MQ} \) rates were obtained at three or four static magnetic fields, ranging from 9.4 Tesla (400 MHz 1H frequency) to 18.8 Tesla (800 MHz 1H frequency).

The CSA values, \( ΔC \) and \( ΔH \), were measured from the 1H-13C dipolar-CSA cross-correlated relaxation rates65-67. For measuring the 13C CSA values, 1H-coupled constant-time heteronuclear SQ coherence spectra were recorded with varied constant-time periods, \( T \), then the relaxation rates of the inner two multiplet components during \( T \) were extracted by fitting the peak intensities to an exponential decay function. The 1H cross-correlated relaxation rates, \( κ_{CH} \), were calculated using equation (9), where \( κ_{CH} = κ_{CH} \) and \( κ_{CH} \) represent the relaxation rates of the up-field and down-field multiplet components, respectively. The \( κ_{CH} \) can be expressed using the methyl threefold axis order parameter, \( S_{ax} \), the rotational correlation time, \( τ_C \), the gyromagnetic ratios, \( γ_C \) and \( γ_H \), the static magnetic field strength, \( B_0 \), the CSA values, \( ΔC \) and \( ΔH \), the relative distance between the HC bond and the methyl threefold axis, \( b \), the length of the methyl 1H-13C bond, \( r_{CH} \), and \( P_{z}(cosθ) \) (3cosθ × 1/2). We used the \( P_{z}(cosθ) \) value of \( 0.228 Å^{-3} \). The \( κ_{CH} \) value of each methyl group was calculated using the \( S_{ax}τ_C \) and the \( κ_{CH} \) rate.

\[ κ_{CH} = \frac{4}{7}\frac{15}{3}\left(S_{ax}τ_C\right)^2 B_0^2 \cdot \frac{P_{z}(cosθ)}{r_{CH}} \]

\[ P_{z}(cosθ) = 0.5x\left(κ_{CH} - κ_{CH} \right) \]

\[ κ_{CH} = \frac{4}{7}\frac{15}{3}\left(S_{ax}τ_C\right)^2 B_0^2 \cdot \frac{P_{z}(cosθ)}{r_{CH}} \]

\[ P_{z}(cosθ) = 0.5x\left(κ_{CH} - κ_{CH} \right) \]

When we used the CSA values, \( ΔC \) and \( ΔH \), to estimate the \( R_{MQ,CSA} \) and \( ΔR_{MQ,CSA} \) rates, the calculated rates tended to be slightly smaller than the experimentally observed rates, probably due to the methyl threefold rotational averaging of the CSA values. Therefore, we used the scaled CSA values, \( ΔC \) and \( ΔH \), in equation (11) when we calculated the \( R_{MQ,CSA} \) and \( ΔR_{MQ,CSA} \) rates. We assume that the principal axes of the 13C and 1H CSA tensors are parallel. As shown in Supplementary Fig. 3c, all four multiplet components were completely resolved in the F1 frequency domain, enabling the comprehensive analysis of the methyl groups. It should be noted that the modified sequence for measuring the \( κ_{CH} \) rate does not contain the purine element, which eliminates the fast-relaxing 1H element48 (Supplementary Fig. 3b). Thus, the use of the original two-dimensional based pulse sequence is recommended for smaller proteins.
The products of the order parameter and the rotational correlation time, $S^2_{\text{rot,CSA}}$, were measured by three-quantum relaxation violated coherence transfer experiments, as established by Sun and co-workers.8,9,68 The intra-methyl $^1H$–$^1H$ dipolar cross-correlated relaxation rates, $\eta_{\text{H-H}}$, were extracted by fitting the peak intensities measured in a pair of data sets to equation (12). $I_1 (T)$ and $I_0 (T)$ represent the peak intensities from two different transfer pathways with the relaxation period $T$, and $\delta$ represents the contributions from external $^1H$ spins.

$$\eta_{\text{H-H}} = \frac{1}{3} \left( \frac{S_{\text{rot,CSA}}}{\delta} \right) \left( \frac{I_{\text{H-H}}}{I_0} \right) \left( \frac{\eta_{\text{H-H}}}{\delta} \right)$$

(12)

The $\eta_{\text{H-H}}$ can be expressed using the methyl three-fold axis order parameter, $S_{\text{rot,CSA}}$, the rotational correlation time, $T_c$, the gyromagnetic ratio, $\gamma$, the angle between the methyl threelfold axis and a vector that connects a pair of methyl $^1H$ nuclei, $\theta_{\text{H-H}}$, the length of the methyl $^1H$–$^1H$ bond, $r_{\text{H-H}}$, and $P_2 (\cos \chi) = (3 \cos^2 \chi - 1)$ (equation (13)). The product of the order parameter and the rotational correlation time, $S_{\text{rot,CSA}}T_c$, was calculated using the $\eta_{\text{H-H}}$ rate.

$$\eta_{\text{H-H}} = \frac{10 \gamma^2 r_{\text{H-H}} P_2 (\cos \chi)}{4 \pi} \left( \frac{S_{\text{rot,CSA}}}{\gamma T_c} \right)$$

(13)

### MQ relaxation analyses of MBP.

To test whether the $\Delta_S$ and $\Delta_{\text{CSA}}$ can be correctly evaluated in high molecular weight proteins, we applied the method to the MBP-$\beta$-cyclodextrin complex (MBP, molecular weight of 42 K), in which the significant chemical exchange processes were reported absent in the majority of the side chain methyl groups.10,11 The complex of MBP (residues 1-370) protein was expressed in E. coli BL21(DE3) cells (Agilent Technologies). For the selective $^{13}C$-labeling of methyl groups, E. coli cells were grown in deuterated M9 media, and 50 mg ml$^{-1}$ of $[^{15}C_2-3$-$^1$H$_2$]-α-ketobutyric acid (for Ile61) (CIL) and 300 mg ml$^{-1}$ of [2-methyl $^{13}$C$_2$-4$^2$H$_3$]-acetate (for Leu62 and Val62) (NMR-Bio) were added 1 h before the induction. The protein was purified sequentially with Q sepharose (GE) and gel filtration column (GE) to remove maltose, and refolded in GuHCl-free buffer (400 MHz) or cryogenic (500 MHz, 600 MHz, 800 MHz) probes. The relaxation delays were varied between 3.9 and 110 ms. The measurements of the chemical exchange processes were obtained using the in-house-developed program written in the programming language, Python 2.7, supplemented with the extension modules, NumPy 1.7 and SciPy 0.11.0. The exchange rate was estimated from the peak intensities with and without the relaxation period $T$. The relaxation delays were varied between 3.9 and 130 ms. The measurements of the chemical exchange rates were performed on Bruker Avance 600 spectrometer equipped with a room temperature probe, using the pulse sequences described in Supplementary Fig. 3a,b. The measurements of the $\eta_{\text{H-H}}$ rates were performed on a Bruker Avance 600 spectrometer equipped with a cryogenic probe. The chemical exchange parameters, using a {u-2H, Ile61, Leu62, Val62}-[1$^{13}$C$_2$]$^{2}$[H$_2$]$_2$] sample, were calculated using equation (13), where $T_{\text{CPMG,ex,CSA}}$ and $T_{\text{CPMG,ex}}$ represent the chemical exchange contributions, and $T_{\text{CPMG,ex}}$ is the chemical exchange process. The MQ-CHEAT method was applied to the chemical exchange processes, and the MQ relaxation analyses were performed on Bruker Avance 400, 500, 600 or 800 spectrometers equipped with room temperature (600 MHz) or a cryogenic (500 MHz, 600 MHz, 800 MHz) probe. The relaxation delays were varied between 3.9 and 110 ms. The measurements of the $\eta_{\text{H-H}}$ were performed on Bruker Avance 600 spectrometer equipped with a room temperature probe, using the pulse sequences described in Supplementary Fig. 3a,b. The measurements of the $\eta_{\text{H-H}}$ rates were performed on a Bruker Avance 600 spectrometer equipped with a room temperature probe. The relaxation delays were varied between 3.9 and 130 ms. The measurements of the chemical exchange rates were performed on Bruker Avance 600 spectrometer equipped with a cryogenic probe.

$$\eta_{\text{H-H}} = \frac{10 \gamma^2 r_{\text{H-H}} P_2 (\cos \chi)}{4 \pi} \left( \frac{S_{\text{rot,CSA}}}{\gamma T_c} \right)$$

(12)

### MQ relaxation analyses of T4 lysozyme mutant.

To validate that the $\Delta_S$ and $\Delta_{\text{CSA}}$ rates can be correctly evaluated, we applied the method to the CS4T, C97A, L99A, G113A, R119P T4 lysozyme mutant (T4L, molecular weight 18 K), which is known to be in a chemical exchange process on a microsecond timescale (refs 68,69).

The Cs4T, C97A, L99A, G113A, R119P T4 lysozyme mutant (T4L, residues 1-164) was expressed in E. coli BL21 (DE3) cells (Agilent Technologies). For the preparation of the [u-$^1$H, Ile61, Leu62, Val62]-[2$^{13}$C$_2$]$^{2}$[H$_2$]$_2$] sample, E. coli cells were grown in deuterated M9 media using [u-$^1$H, $^{13}$C$_2$]-glucose (ISOtec), and 50 mg ml$^{-1}$ of [2-methyl $^{13}$C$_2$-3$^2$H$_2$]-α-ketobutyric acid (for Ile61) (CIL) and 300 mg ml$^{-1}$ of [2-methyl $^{13}$C$_2$-4$^2$H$_3$]-acetate (for Leu62 and Val62) (NMR-Bio) were added both 1 h before the induction. For the chemical exchange processes, we also performed the CPMG RD analyses at 25°C. The chemical exchange processes were obtained using the in-house-developed program written in the programming language, Python 2.7, supplemented with the extension modules, NumPy 1.7 and SciPy 0.11.0. The equation contains four variables used to fit each dispersion curve: the intrinsic transverse relaxation rate $D_1$, the exchange rate $\eta_{\text{H-H}}$, the chemical shift difference $\Delta \sigma$, and the minor state population $p$. Dispersion curves collected at two static magnetic fields were fitted together. The forward and backward rate constants were calculated by extrapolating the linear Arrhenius plot obtained at 4.8, 12 and 16°C. The MQ-CHEAT method was applied to the chemical exchange processes, and the MQ relaxation analyses were performed on Bruker Avance 400, 500, 600 or 800 spectrometers equipped with room temperature (600 MHz) or cryogenic (500 MHz, 600 MHz, 800 MHz) probes. The relaxation delays were varied between 3.9 and 130 ms. The measurements of the $\eta_{\text{H-H}}$ and $\Delta S$ were estimated to be $12,000$ s$^{-1}$ and about 27 s$^{-1}$, respectively, which are comparable to the exchange contributions, $\Delta_{\text{CSA}}$, and can be accurately evaluated in higher molecular weight proteins. The results are consistent with the previous report that significant chemical exchange processes are absent in proteins in crystals.

$$\eta_{\text{H-H}} = \frac{10 \gamma^2 r_{\text{H-H}} P_2 (\cos \chi)}{4 \pi} \left( \frac{S_{\text{rot,CSA}}}{\gamma T_c} \right)$$

(12)
measurements of the \( ^{13}C_{\text{H}} \) rates were performed on a Bruker Avance 600 spectrometer equipped with a room temperature probe. The relaxation delays were varied between 4.0 and 32.0 ms. All NMR experiments were performed at 25 °C.

Supplementary Fig. 6a shows the plots of the \( R_{\text{SQ}} \) and \( D_{\text{MQ}} \) rates of Leu113 as a function of the square of the \( B_0 \). The \( ^{13}C \) SQ CPMG dispersion profile of Leu113 was shown to be dependent on the magnetic field. While the change in the effective relaxation rate (\( R_{\text{eff}} \)) was estimated to be about \( 2 \times 10^{-8} \) s\(^{-1} \) in the CPMG RD analysis, significant magnetic field-dependent changes in the \( R_{\text{SQ}} \) (16 s\(^{-1} \) at 14.1 Tesla) and \( D_{\text{MQ}} \) rates (30 s\(^{-1} \) at 14.1 Tesla) were observed in Leu113. In Leu133, the CSA contributions to the magnetic field-dependent changes were estimated to be about 2 s\(^{-1} \) and, thus the observed magnetic field-dependent change largely originated from the CSA contributions to the magnetic field-dependent changes were estimated to be about 2 s\(^{-1} \) and, thus the observed magnetic field-dependent change largely originated from the chemical exchange contributions. Supplementary Fig. 6b shows the plots of the \( R_{\text{SQ,loc}} \) and \( D_{\text{MQ,loc}} \) rates at 14.1 Tesla (600 MHz \( B_0 \)) frequency which are difficult to detect by CPMG RD analyses. Supplementary Fig. 6c shows the plots of the \( R_{\text{SQ,loc}} \) and \( D_{\text{MQ,loc}} \) rates at 14.1 Tesla (600 MHz \( B_0 \)) frequency which are difficult to detect by CPMG RD analyses. Supplementary Fig. 6d shows the plots of the \( R_{\text{SQ,loc}} \) and \( D_{\text{MQ,loc}} \) rates at 14.1 Tesla (600 MHz \( B_0 \)) frequency which are difficult to detect by CPMG RD analyses. Supplementary Fig. 6e,f show the plots of the \( R_{\text{SQ,loc}} \) and \( D_{\text{MQ,loc}} \) rates at 14.1 Tesla (600 MHz \( B_0 \)) frequency which are difficult to detect by CPMG RD analyses. Supplementary Fig. 6g shows the plots of the \( R_{\text{SQ,loc}} \) and \( D_{\text{MQ,loc}} \) rates at 14.1 Tesla (600 MHz \( B_0 \)) frequency which are difficult to detect by CPMG RD analyses. Supplementary Fig. 6h shows the plots of the \( R_{\text{SQ,loc}} \) and \( D_{\text{MQ,loc}} \) rates at 14.1 Tesla (600 MHz \( B_0 \)) frequency which are difficult to detect by CPMG RD analyses. Supplementary Fig. 6i,j show the plots of the \( R_{\text{SQ,loc}} \) and \( D_{\text{MQ,loc}} \) rates at 14.1 Tesla (600 MHz \( B_0 \)) frequency which are difficult to detect by CPMG RD analyses.

To determine whether the sizes of the obtained \( R_{\text{SQ,loc}} \) and \( D_{\text{MQ,loc}} \) rates, which are difficult to detect by CPMG RD analyses, are consistent with the results from the global fitting procedure, indicating that the \( ^{13}C \) and \( ^{1}H \) chemical exchange parameters obtained from the global fitting procedure with the chemical shift differences calculated from the CPMG RD analyses, except for the \( D_{\text{MQ,ex}} \) rate of Leu118, which is nearly 0 s\(^{-1} \). The slope value, corresponding to \( k_a/p_aB_0 \), is consistent with the value of 2.0 \( \times 10^{-6} \) s\(^{-1} \) and \( p_a = 0.05 \). These results support the proposal that the magnetic field-dependent MQ relaxation analyses are particularly beneficial for detecting chemical exchange processes on a microsecond timescale, which are difficult to detect by CPMG RD analyses.

We constructed 11 Ala mutants (A7V, A11V, A12V, A30V, A31V, A98S, A99S, A101V, A111S, A114V, and A235V), 18 Val mutants (V13A, V50I, V71A, V72I, V73I, V92I, V103I, V111V, V119I, V120I, V131I, V132I, V133I, V134I, V135I, V136I, V137I, V138I, V139I, and V140I), and 18 Ile mutants (I19V, I49V, I55V, I56V, I57V, I62V, I65V, I66V, I67V, I68V, I121V, I122V, I225V, I253V, I264V, I265V, I278V, I285V, I303V, I310V, I315V, and I316V). All mutants were expressed in E. coli MBP, bacteriophage T4 lysozyme are available in the UniProt Knowledgebase under accession codes P08754, P08773, P0AAE9, and P09702. The PDB accession codes 1GGD, 1KKY, 1IDMB and 3DMV were used in this study. The backbone and methyl resonance assignments for Gs-GA and the methyl resonance assignments for Gs-GDI have been deposited in the BMRB with the accession numbers 19013, 26975 and 26976. All other data are available from the corresponding author upon reasonable request.

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Acknowledgements
This work was supported in part by grants from the Japan New Energy and Industrial Technology Development Organization (NEDO) and the Ministry of Economy, Trade and Industry (METI) (to I.S.), a Grant-in-Aid for Scientific Research on Priority Areas from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) (to I.S.), the Development of core technologies for innovative drug development based upon IT from Japan Agency for Medical Research and development, AMED (to I.S.), JSPS KAKENHI Grant Number JP16H01368 (to M.O.), a grant from The Vehicle Racing Commemorative Foundation (to M.O.), and a grant from Nagase Science Technology Foundation (to M.O.).

Author contributions
Y.T., H.K., Y.M., M.O. and I.S. designed the study, Y.T., H.K., Y.M. and M.Y. performed the experiments and Y.T., H.K., Y.M., M.O. and I.S. analysed the data and wrote the paper.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications
Competing financial interests: The authors declare no competing financial interests.
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How to cite this article: Toyama, Y. et al. Dynamic regulation of GDP binding to G proteins revealed by magnetic field-dependent NMR relaxation analyses. Nat. Commun. 8, 14523 doi: 10.1038/ncomms14523 (2017).

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