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An encodable lanthanide binding tag with reduced size and flexibility for measuring residual dipolar couplings and pseudocontact shifts in large proteins

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Abstract

Metal ions serve important roles in structural biology applications from long-range perturbations seen in magnetic resonance experiments to electron-dense signatures in x-ray crystallography data; however, the metal ion must be secured in a molecular framework to achieve the maximum benefit. Polypeptide-based lanthanide-binding tags (LBTs) represent one option that can be directly encoded within a recombinant protein expression construct. However, LBTs often exhibit significant mobility relative to the target molecule. Here we report the characterization of improved LBTs sequences for insertion into a protein loop. These LBTs were inserted to connect two parallel alpha helices of an immunoglobulin G (IgG)-binding Z domain platform. Variants *A* and *B* bound Tb³⁺ with high affinity (0.70 and 0.13 μ M, respectively) and displayed restricted LBT motion. Compared to the parent construct, the metal-bound *A* experienced a 2.5-fold reduction in tag motion as measured by magnetic field-induced residual dipolar couplings and was further studied in a 72.2 kDa complex with the human IgG1 fragment crystallizable (IgG1 Fc) glycoprotein. The appearance of both pseudo-contact shifts (-0.221 to 0.081 ppm) and residual dipolar couplings (-7.6 to 14.3 Hz) of IgG1 Fc resonances in the IgG1 Fc:(variant *A*:Tb³⁺)₂ complex indicated structural restriction of the LBT with respect to the Fc. These studies highlight the applicability of improved LBT sequences with reduced mobility to probe the structure of macromolecular systems.

Introduction

Metal ions exhibit important properties that assist characterization of macromolecular structure and motion. The high electron density of many metals provides unambiguous signals in x-ray diffraction data that can be used to determine phases (Silvaggi et al. 2007). Paramagnetic metals can be observed directly, as contrast reagent, or reveal a wealth of information regarding the structural and dynamic features of macromolecules using electron paramagnetic resonance, magnetic resonance imaging or nuclear magnetic resonance (NMR) spectroscopy (Hiruma et al. 2013; Iwahara and Clore 2006; Pilla et al. 2015; Shulman et al. 1969; Skinner et al. 2015; Tolman et al. 1995). Long distance restraints (5–50 Å) from measurements of paramagnetic effects are invaluable to refining molecular models

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built from short-range nuclear Overhauser effects (NOEs, $<6 \text{ \AA}$) and complementary solution NMR spectroscopy data (Bertini et al. 2002).

Paramagnetic metals can occur naturally in a macromolecule (Butler 1998; Sjødt et al. 2015) or be introduced by replacing an existing metal (Keniry et al. 2006), covalently or non-covalently added as a chelate (Huang et al. 2013; Lee et al. 2015; Loh et al. 2015; Wei et al. 2013; Yang et al. 2015), bound by an unnatural amino acid (Loh et al. 2013; Park et al. 2015), or lanthanide-binding peptide sequence tag (LBT) engineered into the protein sequence (Barthelmes et al. 2011; Feeney et al. 2001; Gaponenko et al. 2000; Martin and Imperiali 2015; Nitz et al. 2003; Su et al. 2008), to reference a few key examples. One common limitation to artificial tags is the presence of metal ion motion. Modeling motion as an additional variable in structure calculations has proven effective with paramagnetic probes including the non-metallic nitroxide spin label and EDTA-Mn(II) complexes (Iwahara et al. 2004a; Iwahara et al. 2004b). For incorporating metals, polypeptide-based tags have the advantage of ease-of-use by simple encoding into a protein expression vector but predicting tag motion for structure calculations is challenging due to an increase in tag size ($\sim 1800 \text{ Da}$) and number of rotatable bonds when compared to the nitroxide labels ($\sim 170 \text{ Da}$). Engineering proteins to attach LBTs within the protein rather than at the N or C termini has proven successful, either through incorporation into a loop connecting two defined secondary structural elements or through secondary attachment to a cysteine residue (Barb et al. 2012; Barthelmes et al. 2011; Saio et al. 2009). Such alterations likely will reduce, but not eliminate, peptide-based tag motion. We sought to improve existing LBT sequences by eliminating unnecessary amino acids that contribute to neither conformational stability nor lanthanide binding.

Paramagnetism from unpaired electrons introduces a strong local magnetic field that increases relaxation rates of nearby nuclei with an r^{-6} distance dependence (paramagnetic relaxation enhancement, or PRE) or perturbs resonance frequencies with an orientation and r^{-3} distance dependence (pseudocontact shifts, or PCSs) in NMR spectra (reviewed in (Koehler and Meiler 2011; Otting 2008)). One advantage of these phenomena is that they are measurable using only 1d or 2d NMR experiments.

Paramagnetic lanthanide ions are becoming increasingly utilized as structural probes because of their limited chemical reactivity, bioorthogonal nature, similar coordination chemistry across the group, and strong magnetic properties. The magnetic susceptibility (χ)-tensor for most paramagnetic lanthanide ions is characterized by significant anisotropy that leads to partial alignment of lanthanide chelates in a magnetic field and, as an independent phenomenon, PCSs of resonances (Otting 2008). Anisotropy is greatest for the Tb^{3+} , Dy^{3+} and Tm^{3+} ions. Though paramagnetic, Gd^{3+} has an isotropic χ -tensor and does not cause alignment or PCSs. The diamagnetic lanthanide ions La^{3+} and Lu^{3+} are useful in control experiments because they neither align nor cause PCSs and PREs.

Partial alignment of a paramagnetic molecule reintroduces residual dipolar couplings (RDCs) that can be used as a powerful mechanism to measure molecule alignment. RDCs are not normally observed in solution NMR experiments due to rapid isotropic tumbling that causes dipolar couplings to average to zero. The coupling strength is related to the distance

between two nuclei and their average orientation with respect to the static magnetic field, thus it is possible to measure the angles between different internuclear vectors in a molecule (Prestegard et al. 2004). The strength of molecular alignment is reflected in the strength of residual dipolar couplings (RDCs) ((Koehler and Meiler 2011; Otting 2008) and references therein). Larger RDC values and a larger principle alignment tensor (D_a) of the alignment indicate a greater degree of alignment. Furthermore, RDCs contribute high-resolution information relating relative domain orientations of multi-domain macromolecules.

Paramagnetic probes are particularly applicable to structural investigations of large systems by solution NMR spectroscopy where molecular mass combined with limited labeling options or conformational exchange favors sparse amino acid labeling and prohibits many modern 3d and 4d experiments to measure long-range through-space and through-bond nuclear interactions (Otting 2008; Yagi et al. 2013). One such system of interest is the Fragment crystallizable (Fc) region of immunoglobulin G1 (IgG1). IgG1 Fc is a homodimer with a single N-glycan at Asn297 that is critical to Fc γ receptor mediated immune cell activation (Hanson and Barb) and glycan composition is strongly correlated with rheumatoid arthritis disease (Parekh et al. 1985). As a result of the post-translational modification, active IgG1 Fc can only be produced in mammalian cells that do not permit deuterium labeling during expression. To probe the IgG1 Fc structure in solution, a chimeric Fc and lanthanide-binding protein was designed based upon three helices of the Fc-binding Z-domain (Nilsson et al. 1987; Tashiro et al. 1997) and a lanthanide-binding tag (LBT) (Nitz et al. 2003; Su et al. 2006), termed Z-I2LBT (Barb et al. 2012). Despite replacing loop 2, connecting helices 2 and 3 of the Z-domain, with the LBT, considerable tag motion was observed (Figure 1).

Here we present the improvement of one polypeptide-based LBT to reduce motion and preserve affinity towards lanthanide ions. Though LBT sequences are applicable to numerous types of techniques, we utilized solution NMR spectroscopy to provide a high-resolution and quantifiable characterization of protein structure and motion. Two variants with reduced loops linking the Z domain to the LBT were prepared and characterized. We also investigated the utility of one design to probe the structure of a large 72.2 kDa complex with IgG1 Fc.

Experimental Conditions

Materials

All materials, unless otherwise noted, were from Sigma-Aldrich. Stable isotope-enriched compounds were purchased from Cambridge Isotopes.

Protein Expression and characterization

Z-I2LBT expression using *E. coli* BL21* cells was described previously (Barb et al. 2012). Open reading frames for variants *A* and *B* were synthesized (Genscript) and cloned into pET29 as described for the Z-I2LBT construct (Barb et al. 2012). Expression, stable isotope labeling, purification, and Tb³⁺ binding measurements of variants *A* and *B* were performed as previously described (Barb et al. 2012). A modified construct of variant *A* was prepared that permitted tobacco etch virus protease (TEV)-catalyzed cleavage of the His tag

(liberating G¹³V¹⁴D¹⁵...K⁸³) for studies of the Fc:A complex. The TEV reaction was performed at 25 °C for 16 h in the dark in a buffer containing 50 mM trisaminomethane, 100 mM sodium chloride, 0.5 mM ethylenediaminetetraacetic acid and 2 mM beta-mercaptoethanol, pH 8.2. The cleaved variant *A* product was isolated by passing the reaction mixture over a Ni-NTA column (Qiagen) and collecting the flow-through fraction. Cleaved variant *A* (1 mL) was then subject to dialysis for 4 h against 1 l of 25 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 100 mM potassium chloride at 25 °C using a 3,000 molecular weight-cutoff dialysis tubing (Spectrum Labs). The dialysis was repeated against a fresh 1 l of the same MOPS buffer.

Expression and purification of the human IgG1 Fc was conducted using HEK293F cells as described (Subedi et al. 2015).

NMR Spectroscopy

NMR spectrometers operating at 21.1 T, 14.1 T (both Varian VNMRs) or 18.8 T, 16.4 T (Bruker Avance 3, Avance 2, respectively) were equipped with cryogenically-cooled 5mm probes. ¹H resonance frequencies were internally referenced to DSS; ¹³C and ¹⁵N frequencies were indirectly referenced using the spectrometer's ¹H frequency at 0 ppm (Markley et al. 1998). NMR data were analyzed using Topspin (Bruker; v.2.1), NMRPipe (Delaglio et al. 1995), NMRViewJ (Johnson and Blevins 1994) and Sparky (version 3.115; Goddard and Kneller, University of California, San Francisco).

Assignment of ¹H-¹⁵N correlation peaks collected using the variant *A*:Lu³⁺ chelate was completed as previously described (Barb et al. 2012) using triple resonance HNCO, HNCA, HNCACB and CBCACONH experiments collected at 14.1T and 10 °C (Cavanagh 2007). Assignments for variant *A* were deposited in the BioMagResBank (BMRB) as accession number 19769. Assignment of residues from the paramagnetic complexes (+Dy³⁺ or +Tb³⁺) were made by comparison to a spectrum of the corresponding diamagnetic complex (+Lu³⁺) and using available backbone resonance assignment data of diamagnetic proteins where applicable (Z-12LBT (BMRB 18126); variant *A* (19769); IgG1 Fc (25224)).

NMR spectra to probe the binding of variant *A* to IgG1 Fc were collected at 16.4 T and 50 °C. ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) spectra of isotopically enriched IgG1-Fc (150 μM: dimer) with selective [¹⁵N]-Tyr amino acid residues were collected with and without variant *A* (330 μM) plus Lu³⁺ (330 μM). NMR samples were prepared in 25 mM MOPS, 100 mM sodium chloride, 0.5 mM DSS and 5% D₂O, pH 7.2.

Measurements of *A* and *B* alignments were made with samples containing 150 μM protein with one molar equivalent of either Lu³⁺ or Dy³⁺ at 25 °C and 21.1 T. NMR spectra to record pseudo-contact shifts (PCS) and residual dipolar coupling measurements (RDCs) on the IgG1 Fc:(variant *A*:Ln³⁺)₂ complex were collected at 18.8 T and 50 °C using 125 μM IgG1 Fc (dimer), 275 μM TEV-cleaved variant *A*, 290 μM Tb³⁺(or Lu³⁺) in a buffer containing 10 mM MOPS, 100 mM KCl, 0.5 mM DSS and 5 % D₂O, pH 7.2. RDCs were measured with a *J*-modulation based strategy as described (Barb et al. 2012; Liu and Prestegard 2009) by subtracting ¹H-¹⁵N couplings measured with diamagnetic samples (containing the diamagnetic Lu³⁺ ion) from couplings measured with paramagnetic samples

(containing the Tb^{3+}) and analyzed as described using PALES (Zweckstetter and Bax 2000). PCS were analyzed using NUMBAT (Schmitz et al. 2008).

Structure figures were prepared using PyMol (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

Results and Discussion

Lanthanide-binding properties of truncated Z-I2LBT variants

Z-I2LBT was targeted for optimization to reduce LBT motion relative to the three Z-domain helices. We designed two variants by eliminating amino acid residues in the two segments connecting the LBT and Z moieties that were believed to facilitate LBT mobility (Barb et al. 2012). Variant *A* (5 residues removed) and variant *B* (2 residues removed) expressed as soluble proteins at high yields using *E. coli* (>100 mg protein / L culture) and were purified (Figure 1).

Variants *A* and *B* bound Tb^{3+} with high affinity in a spectrofluorophotometric assay that excites only polypeptide-bound ions at 280 nm through a Tyr residue in the LBT moiety. Titrating variant *A* or *B* with Tb^{3+} led to saturation of luminescence at 490 and 540 nm and is shown in Figure 2. Variants *A* (700 ± 50 nM) and *B* (130 ± 50 nM) bound with dissociation constants (K_d) that are comparable to that of the original Z-I2LBT protein (130 ± 30 nM (Barb et al. 2012)). As a result of the lanthanide ion binding affinity, either variant will be saturated at a ratio of 1:1 in NMR experiments that typically use >50 μM protein.

Domain motion of the lanthanide-binding Z-I2LBT variants

We estimated field-induced alignments of the Z domain moiety for the *A*: Dy^{3+} and *B*: Dy^{3+} complexes by measuring RDCs. If the Dy^{3+} :LBT moieties of each variant are assumed to align in an identical manner, a reasonable assumption due to complete conservation of the lanthanide-binding residues, principle alignment tensors (Da) fitted by analyzing RDCs of the underlying Z domains may be compared between variants as a relative measurement of Z domain alignment and thus interdomain LBT/Z mobility. This approach does not require the underlying Z-domains to align in the same orientation and was utilized here because the LBT residues were broadened beyond detection due to the paramagnetic ion, eliminating the possibility of measuring the amplitude of interdomain motion directly using RDCs.

HSQC spectra of ^{15}N -labeled variant *A*, variant *B* and Z-I2LBT with a 1 molar equivalent of Dy^{3+} were compared to spectra of diamagnetic Lu^{3+} complexes as shown in Figure 3. The spectrum of variant *A* was affected substantially by PREs in the Dy^{3+} complex with only 14 amide peaks visible. A spectrum of variant *B*+ Dy^{3+} , like the parent Z-I2LBT+ Dy^{3+} complex, contained more than twice as many peaks as *A*. All three spectra showed the effect of PCSs (>0.5 ppm) (the Z-I2LBT spectrum is shown in (Barb et al. 2012)).

A comparison of the one-bond scalar ^{15}N -H coupling ($^1J_{\text{NH}}$) from the unassigned spectra of the designed proteins in a Dy^{3+} complex provided insight into the different molecular properties. Variant *A* displayed the largest range of values (81–114 Hz) while the range of *B* (85–109 Hz) was less than *A* but greater than Z-I2LBT (88–103Hz) as shown in Figure 3A.

The greater range of $^1J_{\text{NH}}$ values and fewer peaks resulting from PREs observed for *A* is consistent with an LBT that is closer to the three Z domain helices and experiences a reduced amplitude of motion.

Measurements of residual ^1H - ^{15}N dipolar couplings for variant *A*, variant *B* and Z-l2LBT with one molar equivalent of Dy^{3+} provided an estimate of LBT motion for the three proteins (Figure 3A). Peaks from *A*+ Dy^{3+} (11) and *B*+ Dy^{3+} (14) were assigned with high confidence and used for further analysis as shown in Figure 3B&C. Alignment tensors (Da) fitted to the RDCs using a model of the Z-domain (pdb 1q2n (Zheng et al. 2004)) were qualitatively consistent with the ranges of ^1H - ^{15}N coupling values (Table 1 and Figure 3A, D&E). Though only a limited number of assigned residues are visible in spectra collected in the presence of Dy^{3+} , these data do provide a means to estimate the motion of each LBT relative to the Z domain helices. The LBT of *A* and *B* experience a range of motion that is estimated to be 0.39 and 0.68 times that of Z-l2LBT, respectively, based on the tensor magnitude. A previous analysis found Z-l2LBT exhibited 40° of interdomain motion using a diffusion-in-a-cone model (Barb et al. 2012; Barbato et al. 1992). By comparison variants *A* and *B* exhibited $\sim 16^\circ$ and 27° of motion, respectively. This analysis, though a simplification that assumes motion is equally distributed within a cone, is informative as a singular estimate of relative mobility between the LBT and Z moieties.

Structural assessment of Z-l2LBT variants

The structural features of these proteins were probed by solution NMR spectroscopy. Both variants *A* and *B* appeared folded and experienced conformational rearrangements upon binding a diamagnetic Lu^{3+} ion based on two-dimensional ^1H - ^{15}N HSQC spectra of variants *A* and *B*, before and after titrating Lu^{3+} (Figure 4). Previous results indicate a significant change in Z-l2LBT conformation upon lanthanide binding is likely limited to the LBT moiety (Barb et al. 2012). Due to the large proportion of the structured protein formed by the LBT (21–23%) and central location it is anticipated that lanthanide binding will induce noticeable chemical shift changes in a majority of the protein as observed (Figure 4). The designed proteins exchanged between lanthanide bound and unbound states in the slow regime of the NMR timescale (data not shown).

A complete backbone resonance assignment of variant *A* assigned all major peaks observed in an ^1H - ^{15}N HSQC spectrum (88% of HN assigned, 89% N, 79% CA, 76% CB, and 73% CO from residues 13–83; Figure 4C) and was consistent with a high degree of structural similarity to the parent Z-l2LBT protein. The high degree of structural similarity is supported by identical functional roles that are dependent upon structure, namely effective Tb^{3+} binding (discussed above) and antibody binding (discussed below). A few residues from loops connecting LBT to helices 2 & 3 plus a few residues from the middle of helix 3 were not observed (Figure 1C).

NMR characterization of the A:Fc complex

We probed binding of variant *A* to IgG1 Fc in order to characterize the effects of two paramagnetic ions in the 72.2 kDa IgG1 Fc:(variant *A*: Tb^{3+})₂ complex (IgG1 Fc is a homodimer with two Z domain binding sites, see Fig 1B). To reduce the rotational

correlation time of the five-component complex and increase sensitivity as much as possible, a variant *A* form was prepared with a TEV-cleavable 6xHis tag. This variant was shown to bind Tb^{3+} with the same high affinity as the variant *A* construct containing a 6xHis tag and the TEV-cleaved form was utilized for subsequent experiments (data not shown).

A comparison of IgG1 Fc spectra before and after the addition of the variant *A*: Lu^{3+} chelate revealed the formation of a tightly bound complex as expected based on affinity of the Z domain for IgG1 Fc (Figure 5) (Barb et al. 2012; Starovasnik et al. 1997). Two IgG1 Fc Tyr crosspeaks shifted as a result of complex formation; Tyr436 is proximal to the Z/Fc interface and experienced a large displacement (Deisenhofer 1981; DeLano et al. 2000). The Tyr373 crosspeak shifted slightly. The remaining six crosspeaks remained in the same spectral position but were slightly less intense likely due to an increase in the rotational correlation time upon variant *A* binding.

Paramagnetic effects of variant *A* in complex with Fc

Strong paramagnetic effects were observed in the IgG1 Fc:(variant *A*: Tb^{3+})₂ complex. Though significant PREs broadened many signals including K248, K288 and K320 as shown in Figure 6, we focused on PCSs and field-induced RDCs of the Fc that indicate structural restriction of the LBT. The presence of PREs is not a good measure of structural restriction because these can be strong even if the tag experiences a high degree of mobility relative to the underlying protein.

Substantial ^1H PCSs were observed in an ^{15}N -HSQC-TROSY spectrum of the IgG1 Fc:(variant *A*: Tb^{3+})₂ complex when compared to the Lu^{3+} -bound complex (Figure 6). Of 21 assignable cross peaks from the Tb^{3+} -bound complex, 20 showed measureable PCSs between -0.221 and 0.081 ppm indicating the LBT tag was stabilized in a position to influence IgG1 Fc resonances. A small degree of heterogeneity is observed with some peaks, including Y436. It is unclear whether the LBT, lanthanide, or Fc contributes to this heterogeneity. A comparison of all observed PCSs with PCSs calculated using a structural model of the Fc monomer (pdb- 1L6X (DeLano et al. 2000)) revealed a high degree of similarity (Figure 6B, the coefficient of determination squared (R^2) = 0.978).

That the PCSs fit the known structure with a high degree of similarity is important for three reasons. First, the Tb^{3+} ion is coordinated near the Fc protein and experiences limited motion, if any, relative to the Fc. Second, the ion is positioned in a manner that preferentially impacts resonances on the nearest monomer, and minimally affects the more distant Fc monomer in the Fc dimer. Third, these data confirm that the 1L6X structural model is a good representation of the Fc conformation in complex with the Z domain and supports recent solution measurements of Fc quaternary structure in solution (Subedi and Barb 2015).

We also pursued RDCs as a measurement of field-induced IgG1 Fc:(variant *A*: Tb^{3+})₂ alignment. We observed six RDCs with a range of -7.6 to 14.3 Hz and error <2.3 Hz (Table 2 and Figure 7). The smaller number of RDCs with low errors (6) with respect to PCS observations (20) reflects the effect of PREs on signal intensity in the *J*-modulation experiments that led to increased errors with the Tb^{3+} sample. These presence of RDCs is

further strong evidence for conformational stability of the variant A LBT tag in the IgG1 Fc complex.

Conclusions

Here we detailed the modification of a chimeric lanthanide and IgG1 binding protein to dramatically reduce the size and mobility of the metal binding moiety. This tagging strategy is widely applicable to other structure-based investigations and has been utilized to study the two-domain SpA-N protein (Prof. T. Oas, Duke University), a two-domain roundabout (ROBO) family construct and a fragment of the extracellular domains of a leukocyte common antigen-related receptor protein tyrosine phosphatase (Prof. K. Moremen, UGA). We demonstrated the utility of one new design to investigate the structure of a large 72.2 kDa complex. These shortened LBTs provide one approach to reducing the motion of LBTs in many other protein systems that require limited LBT motion.

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Figure 1.

Features of chimeric IgG and lanthanide-binding Z domain designs. (A) A green ribbon diagram depicts the Z domain with a lanthanide binding tag in loop 2 (Z-12LBT)(Barb et al. 2012). (B) Z-12LBT binds to the same surface of IgG1 Fc as the parent Z-domain. (C) Variant A and B differ from the parent Z-12LBT protein by removal of 5 and 2 residues from the LBT/Z linker, respectively. Arrows indicate LBT residues that directly interact with Ln^{3+} ions. Underlining of the variant A sequence denotes assigned amide resonances.

Figure 2.
 Tb^{3+} -binding affinity of variants A and B.

Figure 3.

RDCs of the designed proteins. **(A)** The distribution of one-bond ^1H - ^{15}N coupling values for variants A, B, and Z-I2LBT was measured using unassigned peaks in the presence of Dy^{3+} at 21.1 T. **(B, C)** ^{15}N HSQC spectra of variants A and B, respectively, in complex with Dy^{3+} . Pseudo-contact shifts (PCSs) of assigned resonances are shown with a grey line with the origin corresponding to the position in the spectrum collected in the presence of Lu^{3+} . Error bars represent the residue-specific sum of errors for individual fits of the Lu^{3+} and

Dy³⁺ ¹⁵N-H coupling observations. (**D**, **E**) Plots showing the observed RDC values of the *A*:Dy³⁺ and *B*:Dy³⁺ complexes versus the calculated values. The line represents $y = x$.

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Figure 4.

2d NMR spectroscopy of variants A and B. ^{15}N -HSQC spectra of variants A (A) and B (B) before and after addition of Lu^{3+} at 25 °C reveal large changes in protein conformation. (C) Backbone amide crosspeaks of the variant A: Lu^{3+} complex were assigned with standard triple-resonance experiments collected at 10 °C. Sidechain amides are identified with horizontal grey lines.

Figure 5. ^{15}N -HSQC spectra of ^{15}N -Tyr-IgG1 Fc collected at 16.4 T before and after addition of two molar equivalents of variant A and Lu^{3+} . Amino acid assignments are based on a published report (Matsumiya et al. 2007).

Figure 6.

Pseudo-contact shifts (PCSs) were detected among the [^{15}N -Tyr/Lys]-IgG1 Fc amide resonances at 18.8 T. **(A)** An overlay of ^{15}N -HSQC-TROSY spectra collected using the IgG1 Fc(Z-I2LBT:Lu $^{3+}$) $_2$ and IgG1 Fc(Z-I2LBT:Tb $^{3+}$) $_2$ complexes are shown in *black* and *orange* contours, respectively. Significant differences in resonance frequencies are denoted with *white and grey* arrows. **(B)** The observed ^1H PCSs correlate strongly to ^1H PCSs calculated by the NUMBAT program (Schmitz et al. 2008), using a model of the Fc protein determined by x-ray crystallography (DeLano et al. 2000). A line corresponding to the function $y = x$ is shown in *black*.

Figure 7. Residual dipolar couplings (RDCs) of the Fc amide resonances were identified using the IgG1 Fc(Z-I2LBT:Tb³⁺)₂ complex. Spectra collected using isotropic (*black* line with *black* circles; complex with Lu³⁺) and partially aligned (*orange* line with *open* circles; complex with Tb³⁺) conditions at 18.8 T revealed significant positive and negative RDCs using a *J*-modulation method to measure ¹H-¹⁵N couplings (Liu and Prestegard).

Table 1
RDC alignment parameters for Z-I2LBT, and variants *A* and *B* in complex with Dy³⁺ at 21.1T.

Protein	# Obs	Da	Dr	Theoretical Max Coupling (Hz)	Q- value(Cornilescu et al. 1998)
Z-I2LBT	20	2.9×10^{-4}	9.0×10^{-5}	6.2	0.317
Variant <i>B</i>	14	4.2×10^{-4}	2.5×10^{-4}	9.1	0.261
Variant <i>A</i>	11	7.3×10^{-4}	4.2×10^{-4}	15.8	0.178

Table 2
Field-induced RDCs from IgG1 Fc residues in the IgG1 Fc:(variant A:Tb³⁺)₂ complex

residue	Coupling with Lu ³⁺ (Hz)		Coupling with Tb ³⁺ (Hz)		RDC (Hz)
		Error (Hz)		Error (Hz)	
K290	92.4	1.4	106.7	2.2	14.3
Y349	97.2	1.2	95.3	1.5	-1.9
K360	95.7	2.1	88.1	0.8	-7.6
Y391	96.0	2.0	94.9	1.3	-1.1
K392	97.4	2.3	105.9	2.2	8.5
Y407	95.8	2.3	100.4	0.8	4.6