Supporting Information

Unraveling a Ligand-Induced Twist of a Homodimeric Enzyme by Pulsed Electron–Electron Double Resonance

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1 Preparation and characterization of Zymomonas mobilis TGT variants

1.1 Mutagenesis

Mutagenesis of the Z. mobilis \textit{tgt} gene was performed as previously described by site-directed mutagenesis and plasmid-based expression of the vector pPR-IBA2 (IBA Lifesciences).\cite{1} Plasmids coding the required mutated TGT variants (see below) were prepared using the PureYield\textsuperscript{TM} Plasmid Miniprep System (Promega). Based on the TGT(F92C/C158S/C281S) construct\cite{1}, a reverse C92F and single-site surface cysteine mutations were introduced via the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) according to the vendor instructions. DNA primers (Table S1) were purchased from Eurofins Genomics. In each case, the new construct was re-sequenced by Eurofins Genomics to confirm the presence of the desired mutations as well as the absence of any further unwanted mutation. The resulting constructs were each transformed into \textit{Escherichia coli} BL21-CodonPlus (DE3)-RIPL cells (Agilent). A single colony was picked and grown overnight in 100 mL of lysogeny broth (LB) media supplemented with 100 μg mL$^{-1}$ ampicillin and 34 μg mL$^{-1}$ chloramphenicol while shaking at 37 °C.

Table S1. DNA primer sequences used for mutagenesis. Nucleobases deviating from the original \textit{tgt} sequence are underlined.

| Mutation / primer ID | Sequence |
|----------------------|----------|
| G87C\text{\textunderscore}f | 5'-GTATTGCAAAACTGTGTGCTCTGATACG-3' |
| G87C\text{\textunderscore}r | 5'-GCTATGCAGACCACAATTTTGCAATAC-3' |
| H319C\text{\textunderscore}f | 5'-GGATAGCGAATGTGCACATTTGATGCATAC-3' |
| H319C\text{\textunderscore}r | 5'-GACAAACTGCACAGCACATTGATGCTATCC-3' |
| C92F\text{\textunderscore}f | 5'-GTGGTCTGAGTCTATGGGTGAGATTC-3' |
| C92F\text{\textunderscore}r | 5'-GATCCCAACCCATAAGCTATGAGCAGACCAC-3' |
1.2 Preparation of spin-labeled TGT variants

For overexpression of the TGT mutants, transformed cells were transferred and grown overnight in 100 mL of LB media supplemented with 100 μg mL⁻¹ ampicillin and 34 μg mL⁻¹ chloramphenicol while shaking at 37 °C. The next day, a large-scale culture in LB medium, supplemented with 100 μg mL⁻¹ ampicillin and 34 μg mL⁻¹ chloramphenicol, was inoculated with 10 mL of the overnight culture per liter. The culture was grown at 37 °C by shaking at 140 rpm until the optical density at 600 nm reached a value between 0.6 and 0.7. After cooling to 16 °C, overexpression was induced by the addition of IPTG to a final concentration of 1 mM. The cells were harvested by centrifugation using a JA-10 rotor (Beckman Coulter) at 4 °C and 10000 rpm for 10 min after a total expression time of ~18 h. The collected cells were stored at −80 °C until further usage.

For purification, the cells were disrupted by sonication using a Branson Sonifier™ 250 in 100 mL lysis buffer (20 mM TRIS, pH 7.8, 10 mM EDTA, 1 mM DTT and 1 cOmplete™ Protease Inhibitor Cocktail Tablet (Roche) per 4 L of bacterial culture). Subsequently, the cell debris was removed by centrifugation using a JA-25.50 rotor (Beckman Coulter) at 4 °C and 20000 rpm for 1 h. The supernatant was collected and loaded onto a Q Sepharose® Fast Flow anion exchange column (XK 26/15; GE Healthcare) pre-equilibrated with buffer A (10 mM TRIS, pH 7.8, 1 mM EDTA, 1 mM DTT). After washing with buffer A, the target protein was eluted by applying a NaCl gradient from 0 to 100 % (v/v) buffer B (10 mM TRIS, pH 7.8, 1 mM EDTA, 1 mM DTT, 1 M NaCl). The protein-containing fractions, endowed with an N-terminal Strep-tag® II, were then loaded onto a Strep-Tactin® Superflow® column (IBA Lifesciences) conditioned with buffer W (100 mM TRIS, pH 7.8, 1 mM EDTA, 1 M NaCl). After washing the column with buffer W, the protein was eluted with buffer E (100 mM TRIS, pH 7.8, 1 mM EDTA, 1 M NaCl, 2.5 mM D-desthiobiotin). All chromatographic steps were carried out at room temperature using an ÄKTApPrime™ plus FPLC system (GE Healthcare). All protein-containing fractions were combined and concentrated to ~2 mg mL⁻¹ in high-salt buffer (10 mM TRIS, pH 7.8, 1 mM EDTA, 2 M NaCl) using Vivaspin® 20 centrifugal concentrators (30000 MWCO, Sartorius). Subsequently, the Strep-tag® II was chipped off and separated from the target protein using the Thrombin Cleavage Capture Kit (Novagen®) according to the manufacturer’s instructions.

After tag cleavage, the protein was spin-labeled by incubation with 10 eq. of MTSL overnight at room temperature. In case of TGT(H319R1), prior to spin labeling, the protein was treated with 10 mM DTT for 2 h at room temperature. Subsequently, DTT was removed by filtering through an illustra™ NAP-25 column (GE Healthcare). In contrast, the TGT(G87R1) variant
was spin labeled without prior DTT treatment. Spin labeling efficiencies were determined by mass spectrometry (Figure S1). The next day, the sample was filtered through an illustra™ NAP-25 column (GE Healthcare) pre-equilibrated with high-salt buffer to remove unbound MTSL. Prior to the PELDOR experiments, the sample was dialyzed against deuterated PELDOR buffer (50 mM TES, pH 7.4, 2 M NaCl in D2O) and concentrated using Vivaspin® 20 centrifugal concentrators (30000 MWCO, Sartorius) to a final TGT monomer concentration of 120 µM. In case of the ligand-bound TGT samples, 3 eq. of each of the ligands 1–4 (from 10 mM stock in DMSO) or 1.5 eq. of tRNA (from 3 mM stock in the deuterated PELDOR buffer) were added to the protein to a final sample volume of 100 µL. If not mentioned explicitly, the samples were incubated for 24 h and then transferred into an EPR tube and flash frozen in liquid nitrogen until further usage.

1.3 Preparation of tRNA
The *E. coli* tRNA\textsubscript{Tyr} (ECY2\textsuperscript{[2]}) was synthesized by *in vitro* transcription using T7 RNA polymerase. The reaction mixture (30 µg of linearized DNA template, 3.75 mM of NTPs, and 1 µM of T7 RNA polymerase in 20 mM MgCl\textsubscript{2}, 80 mM HEPES, pH 7.5, 1 mM spermidine, 5 mM DTT, 0.05 U pyrophosphatase) was incubated for 4 h at 37 °C. The tRNA transcript was extracted in a 1:1 mixture of acidic phenol/chloroform, pH 4.5, and precipitated upon addition of a 1:20 mixture comprising 3 M sodium acetate, pH 5.2, and ethanol. After centrifugation for 10 min at 16500 g and 4 °C, the supernatant was discarded. The pellet was dried, dissolved in ddH\textsubscript{2}O, and the tRNA was purified via preparative denaturing urea polyacrylamide gel electrophoresis (8 M urea, 8 % polyacrylamide). The tRNA was eluted from the excised gel slice in 1 M sodium acetate, pH 5.2, overnight while shaking at 900 rpm and 4 °C. The purified tRNA was again precipitated using the procedure described above and finally dried *in vacuo*. 
Figure S1. Intact mass spectra of a) TGT(G87R1/C158S/C281S) (calculated mass: 43212.1 Da (labeled), 43027.8 Da (unlabeled)) and b) TGT(C158S/C281S/H319R1) (calculated mass: 43132.0 Da (labeled), 42947.7 Da (unlabeled)).
1.4 Kinetic characterization

The kinetic parameters, $k_{\text{cat}}$ and $K_m$(tRNA$^{\text{Tyr}}$), of the spin-labeled TGT variants were determined by monitoring the insertion of [8-$^3$H]-guanine (1.5 Ci mmol$^{-1}$, American Radiolabeled Chemicals, Inc.) into tRNA as described by Biela et al.$^{[3]}$ In each case, the enzyme was used at a subunit concentration of 150 nM. While the concentration of [$^3$H]-labeled guanine was kept constant at 10 μM, the concentration of tRNA$^{\text{Tyr}}$ varied between 0.26 and 15 μM. Plots were generated and kinetic parameters were calculated using GraphPad Prism 6 (Figure S2 and Table S2).

![Graphs showing Michaelis-Menten plots and progress curves for TGT variants](image)

**Figure S2.** Michaelis-Menten plots (left) and corresponding progress curves (right) of a) TGT(G87R1/C158S/C281S) and b) TGT(C158S/C281S/H319R1).

| TGT variant                  | $K_m$(tRNA$^{\text{Tyr}}$) [μM] | $k_{\text{cat}}$ [$10^{-3}$ s$^{-1}$] |
|------------------------------|-------------------------------|-------------------------------------|
| wild-type TGT               | 0.8 ± 0.1$^{[4]}$             | 9.0 ± 0.3$^{[4]}$                  |
| TGT(G87R1/C158S/C281S)      | 2.1 ± 0.2                     | 10.3 ± 0.3                         |
| TGT(C158S/C281S/H319R1)     | 2.1 ± 0.2                     | 11.8 ± 0.3                         |

**Table S2.** Kinetic parameters of TGT variants.
1.5 Crystallization and structure determination

Crystallization of TGT was performed using the hanging-drop vapor diffusion method at 18 °C. For the R1-labeled TGT variants, the protein solutions (10 mg mL⁻¹ in 10 mM TRIS, pH 7.8, 1 mM EDTA, 2 M NaCl) were each mixed with reservoir solution (100 mM TRIS, pH 8.5, 10 % (v/v) DMSO, 7 % (w/v) PEG 8000 or 100 mM MES pH 5.5, 0.5 mM DTT, 10 % (v/v) DMSO, 13 % (w/v) PEG 8000) in a 1:1 volume ratio. In case of the co-crystallization of functional wild-type (WT) TGT with ligand 2 (TGT-2), the protein solution (31 mg mL⁻¹ in 10 mM TRIS, pH 7.8, 1 mM EDTA, 2 M NaCl) was mixed with DMSO stock of ligand 2 yielding a final concentration of 1.6 mM. The protein–inhibitor mixture was then mixed with reservoir solution (100 mM MES pH 5.5, 0.5 mM DTT, 10 % (v/v) DMSO, 13 % (w/v) PEG 8000) in a 1:2 volume ratio. Crystals grew within two weeks in the presence of 500 or 650 μL of reservoir solution in the plate wells. Prior to data collection, the crystals were transferred to a reservoir buffer solution containing 30 % (v/v) glycerol as cryo-protectant for a few seconds and vitrified in liquid nitrogen. Diffraction data were collected at a wavelength of 0.91841 Å and temperature of 100 K at the synchrotron beamline 14.1 at BESSY II (Helmholtz-Zentrum Berlin). Indexing, processing and scaling of the diffraction images were done using XDS⁵ and XDSAPP⁶. The structures were determined via molecular replacement using the program Phaser⁷ from the CCP4 suite⁸ with the PDB entry 1P0D or 1PUD as initial search model. Model building was done in Coot⁹ and the program PHENIX¹⁰ was used for structure refinement. Data collection and refinement statistics are summarized in Table S3.
Table S3. Crystallographic data collection, processing, and refinement statistics.

| Protein          | TGT-2 in C2 | TGT(G87R1/C158S/C281S) | TGT(C158S/C281S/H319R1) |
|------------------|-------------|------------------------|--------------------------|
| PDB ID           | 6YGR        | 7APL                   | 7APM                     |
| **(A) Data collection and processing** |             |                        |                          |
| Collection site  | BESSY II MX 14.1 | BESSY II MX 14.1 | BESSY II MX 14.1 |
| No. of crystals used | 1           | 1                      | 1                        |
| Wavelength [Å]   | 0.91841     | 0.91841                | 0.91841                  |
| Space group      | C2          | C222 1                 | C2                       |
| **Unit cell parameters** |           |                        |                          |
| a, b, c [Å]      | 83.92 65.02 71.10 | 64.54 90.95 167.82 | 91.91 65.06 70.81 |
| α, β, γ [°]      | 90.0 93.9 90.0 | 90.0 90.0 90.0 | 90.0 96.3 90.0 |
| **(B) Overall diffraction data (values for highest resolution shell in parentheses)** | | | |
| Resolution range [Å] | 41.86–1.70 | 44.59–1.99 | 45.68–1.66 |
| No. of unique reflections | 40707 (6285) | 35628 (5311) | 47688 (7302) |
| R_{sym} [%]      | 5.1 (49.9)  | 6.3 (49.8)  | 7.5 (46.6) |
| Completeness [%] | 97.5 (93.4) | 97.9 (97.6) | 97.6 (92.8) |
| Multiplicity     | 3.4 (3.4)   | 5.1 (5.6)   | 3.4 (3.2)   |
| Mean I/σ        | 15.3 (2.2)  | 15.2 (2.8)  | 10.4 (2.1)  |
| R_{meas} [%]     | 6.1 (59.3)  | 7.0 (55.0)  | 9.0 (56.0)  |
| CC/1/2          | 99.9 (80.2) | 99.8 (88.7) | 99.7 (82.0) |
| Matthews coefficient [Å³ Da⁻¹] | 2.3        | 2.9        | 2.4         |
| Solvent content [%] | 45.4      | 56.8       | 49.5       |
| Wilson B-factor [Å²] | 21.8     | 33.8       | 18.5       |
| No. of proteins in asymmetric unit | 1         | 1          | 1           |
| **(C) Refinement with PHENIX**[10] (version 1.16_3549) | | | |
| Resolution range [Å] | 41.86–1.71 | 44.59–1.99 | 45.68–1.66 |
| No. of used reflections | 40699     | 33624       | 47683       |
| Final R values |                                             |             |             |
| R_{work} [%]      | 18.2        | 17.2        | 13.9        |
| R_{free} [%]      | 22.1        | 20.9        | 17.6        |
| No. of atoms (non-hydrogen) |          |             |             |
| Protein atoms     | 2698        | 2913        | 2960        |
| Water molecules   | 215         | 173         | 319         |
| Ligand atoms      | 23          | -           | -           |
| RMSD from ideality |                        |             |             |
| Bond length [Å]   | 0.006       | 0.006       | 0.008       |
| Bond angles [°]   | 0.8         | 0.8         | 0.9         |
| Ramachandran plot[4] |                       |             |             |
| Most favored regions [%] | 94.7       | 94.4        | 94.4        |
| Additionally allowed regions [%] | 5.0       | 5.0         | 5.0         |
| Generously allowed regions [%] | 0.3       | 0.6         | 0.6         |
| Mean B-factors [Å²] |                        |             |             |
| Protein atoms     | 25.9        | 42.5        | 22.9        |
| Water molecules   | 33.5        | 44.7        | 31.8        |
| Ligand atoms      | 21.7        | -           | -           |

\[ a \ R_{sym} = \frac{\sum_{hkl} \sum_{i} |I_{hkl},i - \langle I_{hkl} \rangle|}{\sum_{hkl} \sum_{i} I_{hkl},i}, \quad R_{\text{meas}} = \frac{\sum_{hkl} \sqrt{\frac{n}{n-1} \sum_{i} |F_{o,hkl,i} - F_{c,hkl,i}|}}{\sum_{hkl} \sqrt{\sum_{i} F_{o,hkl,i}}} \] [11]

\[ b \ R_{\text{work}} = \frac{\sum_{hkl} |F_{o,hkl,i} - F_{c,hkl,i}|}{\sum_{hkl} F_{o,hkl,i}} \] [11]

\[ c \ R_{\text{free}} \text{ was calculated as } R_{\text{work}} \text{ but on 5\% of the data excluded from the refinement.} \]

\[ d \ Calculated via PROCHECK[12] \]

\[ e \ Calculated via MOLEMAN[13] \]
1.6 Analytical gel filtration

Analytical size exclusion chromatography was performed using an ÄKTAprime™ plus FPLC system (GE Healthcare) at room temperature. The protein samples were diluted to 10 µM in assay buffer (10 mM HEPES, pH 7.4, 1 M NaCl) and 100 μL of each sample was loaded onto a pre-equilibrated Superdex™ 200 10/300 GL column (GE Healthcare). The samples were run at a flowrate of 0.5 mL min⁻¹ and protein absorbance was tracked at 280 nm wavelength. Data were processed with GraphPad Prism 6. The TGT(Y330D) variant has been previously investigated via native mass spectrometry showing the presence of the monomeric species in 99 % at 10 µM protein concentration.[1]

Figure S3. a) Structural comparison of functional wild type TGT homodimer (gray, PDB-ID 1P0D) with TGT(G87R1) (orange, PDB-ID 7APL) and TGT(H319R1) (blue, PDB-ID 7APM). Labeling positions are highlighted in subpanels with R1-label side chains shown as sticks and corresponding electron densities from 2mF₀−DF₀ map contoured at 1.0 σ. b) Gel filtration elution profiles of TGT variants at 10 µM protein concentration. c), d) MtsslWizard-based predictions of the G87R1-G87R1 and H319R1-H319R1 distances for functional WT TGT homodimer (gray, PDB-ID 1P0D), TGT(G87R1) (orange, PDB-ID 7APL), and TGT(H319R1) (blue, PDB-ID 7APM). The H319R1-H319R1 distance determined from the crystal structure TGT(H319R1) is shown by the magenta line.
2 Search for the best spin labeling positions

Availability of the crystal structures for both protein states, the functional state and the twisted form, for TGT-1 (PDB-IDs 5LPS and 5LPT) and TGT-2 (PDB-IDs 6YGR and 5I07) allowed the systematic search for the best spin labeling positions. This search was done by means of the program MtsslWizard, which allows modeling the conformational ensembles of spin labels attached to the protein and to calculate the distances between these ensembles. All possible spin labeling positions in TGT were ranked based on the following three criteria:

1) The spin labeling site has to be accessible for the spin label in both protein states. This is necessary for obtaining good labeling efficiency and also minimizes the probability of the local structural changes of the protein due to the introduction of the spin label.

2) The distance between two spin-labeled sites within each TGT homodimer, $r$, should be in the range of 15–60 Å in both protein states. This ensures that $r$ is in the working range of PELDOR.

3) The distance $r$ should differ between the functional and twisted states by more than 5 Å. This allows distinguishing both protein states through the difference of the corresponding inter-label distances.

The algorithm and the results of the search for both TGT-1 and TGT-2 are depicted in Figure S4. As can be seen, only few residues fulfil all three criteria. These include the residues 36, 87, 91, 234, 236, 301, 304, 305, 306, and 319 for TGT-1 and the residues 36, 63, 87, 91, 160, 234, 236, 286, 304, 305, and 306 for TGT-2. Two of them, namely 87 and 319, were used for the PELDOR experiments in the present study.
Figure S4. Search for the best spin labeling positions in TGT using the program MtsslWizard. a) The search algorithm. b) The results of the search for TGT-1 (PDB-IDs 5LPS and 5LPT). c) The results of the search for TGT-2 (PDB-IDs 6YGR and 5I07). $r$ denotes the distance between two spin-labeled sites within each TGT homodimer. $\Delta r$ denotes the difference in $r$ between the functional and twisted states of TGT. Line breaks indicate residues ill-defined in the electron density of the used protein models.
3 PELDOR measurements and data analysis

3.1 PELDOR measurements

The PELDOR measurements were carried out on an ELEXSYS E580 (Bruker) spectrometer using a FlexLine probe head with a Q-band resonator ER5106QT-2 (Bruker). All microwave pulses were amplified via a 150 W TWT amplifier (model 187Ka). To obtain the working temperature of 50 K, a continuous flow helium cryostat CF935 (Oxford Instruments) and a temperature control system iTC 503S (Oxford Instruments) were employed.

The PELDOR experiments were performed with the standard four-pulse sequence \( \pi/2(v_{\text{det}})-\tau_1-\pi(v_{\text{det}})-\tau_2-\pi(v_{\text{pump}})-\tau_2-\pi(v_{\text{det}})\)-echo. The frequency of the pump pulse and the magnetic field were adjusted to be on resonance with the maximum of the nitroxide spectrum, whereas the frequency of the detection pulse was set 100 MHz lower than the frequency of the pump pulse. All PELDOR measurements were performed with pulse lengths of 12 and 24 ns for the detection \(\pi/2\)- and \(\pi\)-pulses, respectively. The pump pulse was 14 ns long. The detection \(\pi/2\)-pulse was phase-cycled to eliminate receiver offsets. The \(\tau_1\) interval was set to a starting value of 240 ns and was incremented during each experiment 8 times with a step of 16 ns, in order to suppress deuterium Electron Spin Echo Envelope Modulation (ESEEM) artefacts. The \(\tau_2\) interval was set to 5–7 \(\mu\)s. The position of the pump pulse relative to the primary echo was incremented with a step of 8 ns. All PELDOR spectra were recorded at 50 K with a repetition time of 3 ms. The signal was averaged over 50–200 runs to achieve a good signal-to-noise ratio.

3.2 PELDOR data analysis

The PELDOR time traces were converted into the distance distributions using the program DeerAnalysis (version 2019)\(^{[14]}\). Within DeerAnalysis two different protocols were used: 1) Tikhonov regularization with the L-curve criterion\(^{[14]}\), which is the most common approach nowadays, and 2) the DEERNet approach, based on neural networks\(^{[15]}\). Prior to the Tikhonov regularization, the original PELDOR time traces were background corrected using a three-dimensional homogeneous background. The starting position for the background correction was set to the value optimized by DeerAnalysis. Then, Tikhonov regularization was applied using the L-curve criterion. The optimized regularization parameter fell in the range of 100–10000. The 2\(\sigma\) errors of the distance distributions obtained by Tikhonov regularization were estimated using the Validation tool of DeerAnalysis2019. During validation, the noise level and the starting position for the background correction were varied in the range [1.0, 1.5] (10 trials) and [1 \(\mu\)s, 4 \(\mu\)s] (50 trials), respectively. This resulted in a total of 500 trials, for which the distance
distribution was computed using the Tikhonov regularization with the pre-optimized regularization parameter.

In the DEERNet approach, the background of the original PELDOR time traces are modeled by a stretched exponential function. The background correction is integrated into the main analysis i.e., the background does not need to be removed from the original PELDOR time traces before their conversion to distance distributions. For the PELDOR time traces of TGT(G87R1) and TGT(WT)/TGT(G87R1), DEERNet was run using the default “generic” library. For the PELDOR time traces of TGT(H319R1) and TGT(G87R1)/TGT(H319R1), the “generic” library was replaced by the “narrow” library, because the latter one yielded better fits to the experimental time traces. The 2σ errors of the distance distributions obtained by DEERNet were calculated using an ensemble of independently trained neural networks.

3.3 Calculation of relative amounts of the functional and twisted TGT dimers from PELDOR-based distance distributions

To determine the relative amounts of the functional and twisted dimers in the TGT samples, the integrals of the corresponding distance peaks in the PELDOR distance distributions were evaluated. For the TGT(G87R1) variant, the distance intervals of [10, 40] Å and [40, 70] Å were assigned to the twisted and functional dimers, respectively. Inverted intervals were used for the TGT(H319R1) variant.

As described in Section 3.2, each PELDOR time trace was translated into a distance distribution using two approaches, Tikhonov regularization and DEERNet. Therefore, the integral values were evaluated twice for each TGT sample. In both cases, the uncertainties of the PELDOR-based distance distributions were taken into account. We only considered those distance peaks as being real that showed intensity beyond the uncertainty range. The uncertainty of these peaks was then used to estimate the errors of the calculated integrals and the corresponding relative amounts of the dimers. First, the integral values were computed for the lower and upper bounds of the uncertainty ranges determined for each distance distribution. This yielded two integral values for the functional dimer, \( I_{\text{low}}(\text{functional}) \) and \( I_{\text{high}}(\text{functional}) \), and two integral values for the twisted dimer, \( I_{\text{low}}(\text{twisted}) \) and \( I_{\text{high}}(\text{twisted}) \), per distance distribution. Second, the minimal and the maximal ratios between the functional and twisted dimers were calculated as \( I_{\text{low}}(\text{functional})/I_{\text{high}}(\text{twisted}) \) and \( I_{\text{high}}(\text{functional})/I_{\text{low}}(\text{twisted}) \), respectively.
3.4 Calculation of PELDOR modulation depths

Processing of PELDOR time traces by DeerAnalysis, using either Tikhonov regularization or DEERNet, allows determining the modulation depth parameters \( \lambda \) for the time traces. The error of the computed \( \lambda \) is related to slightly different experimental conditions, e.g., in the excitation bandwidth of the pump pulse, and errors in the background correction. We performed technical repeats showing that the experimental error in \( \lambda \) is on the order of 1\% if the pump pulse is carefully adjusted to be an inversion pulse of the same length. The error related to the background correction was computed using all background trials generated at the validation stage of the Tikhonov regularization analysis or by DEERNet. We were not able to perform biological repeats beyond the ones we give in the manuscript, due to the very elaborate multi-step synthesis of the ligands and, therefore, their very limited amounts.
4 PELDOR measurements on TGT(G87R1) and TGT(H319R1)

Figure S5. Original PELDOR time traces of a) TGT(G87R1) and b) TGT(H319R1) without and with ligands.
Figure S6. Processing of PELDOR time traces of TGT(G87R1) without and with ligands. The results of Tikhonov regularization and DEERNet are shown in red and green, respectively. a) Original PELDOR time traces and their background fits. b), c) Background-corrected PELDOR time traces and their fits. d) PELDOR-derived inter-nitroxide distance distributions. Light red and light green shades depict the error estimates for the distance distributions.
Figure S7. Processing of PELDOR time traces of TGT(H319R1) without and with presence of ligands. The results of Tikhonov regularization and DEERNet are shown in red and green, respectively. a) Original PELDOR time traces and their background fits. b), c) Background-corrected PELDOR time traces and their fits. d) PELDOR-derived inter-nitroxide distance distributions. Light red and light green shades depict the error estimates for the distance distributions.
Table S4. Relative amounts of functional and twisted TGT dimers (in %).

| Sample          | Functional dimer* | Twisted dimer*          |
|-----------------|-------------------|-------------------------|
|                 | Tikhonov          | DEERNet                 | Tikhonov          | DEERNet         |
|                 | regularization    |                         | regularization    |                 |
| TGT(G87R1)      | 100               | 100                     | 0                 | 0               |
| TGT(G87R1)-tRNA | 100               | 100                     | 0                 | 0               |
| TGT(G87R1)-4    | 100               | 100                     | 0                 | 0               |
| TGT(G87R1)-3    | 100               | 100                     | 0                 | 0               |
| TGT(G87R1)-2    | 88±6              | 97±2                    | 12±7              | 3±2             |
| TGT(G87R1)-1    | 18±7              | 19±6                    | 82±7              | 81±7            |
| TGT(H319R1)     | 100               | 100                     | 0                 | 0               |
| TGT(H319R1)-tRNA| 100               | 100                     | 0                 | 0               |
| TGT(H319R1)-4   | 100               | 100                     | 0                 | 0               |
| TGT(H319R1)-3   | 100               | 100                     | 0                 | 0               |
| TGT(H319R1)-2   | 92±7              | 96±2                    | 8+14              | 4+3             |
| TGT(H319R1)-1   | 16±10             | 13±1                    | 84±10             | 87±11           |

a The errors were determined in accordance to Section 3.3.

Table S5. PELDOR modulations depth parameters.

| Sample          | Modulation depth parameter (λ)* |
|-----------------|---------------------------------|
|                 | Tikhonov regularization | DEERNet       |
| TGT(G87R1)      | 0.35±0.06                  | 0.34±0.00     |
| TGT(G87R1)-tRNA | 0.33±0.04                  | 0.32±0.01     |
| TGT(G87R1)-4    | 0.33±0.07                  | 0.31±0.01     |
| TGT(G87R1)-3    | 0.32±0.06                  | 0.29±0.01     |
| TGT(G87R1)-2    | 0.35±0.02                  | 0.30±0.00     |
| TGT(G87R1)-1    | 0.31±0.03                  | 0.31±0.01     |
| TGT(H319R1)     | 0.33±0.01                  | 0.32±0.00     |
| TGT(H319R1)-tRNA| 0.31±0.02                  | 0.29±0.01     |
| TGT(H319R1)-4   | 0.27±0.00                  | 0.29±0.00     |
| TGT(H319R1)-3   | 0.32±0.02                  | 0.33±0.00     |
| TGT(H319R1)-2   | 0.33±0.03                  | 0.32±0.00     |
| TGT(H319R1)-1   | 0.29±0.06                  | 0.27±0.01     |

a The errors were determined in accordance to Section 3.4.
Figure S8. Processing of PELDOR time traces of TGT(G87R1) incubated over 1, 24, and 72 h with 1. The results of Tikhonov regularization and DEERNet are shown in red and green, respectively. a) Original PELDOR time traces and their background fits. b), c) Background-corrected PELDOR time traces and their fits. d) PELDOR-derived inter-nitroxide distance distributions. Light red and light green shades depict the error estimates for the distance distributions.
Figure S9. Processing of PELDOR time traces of TGT(G87R1) incubated over 1, 24, and 72 h with 2. The results of Tikhonov regularization and DEERNet are shown in red and green, respectively. a) Original PELDOR time traces and their background fits. b), c) Background-corrected PELDOR time traces and their fits. d) PELDOR-derived inter-nitroxide distance distributions. Light red and light green shades depict the error estimates for the distance distributions.
Figure S10. Processing of PELDOR time traces of TGT(G87R1) incubated over 1, 24, and 72 h with 3. The results of Tikhonov regularization and DEERNet are shown in red and green, respectively. a) Original PELDOR time traces and their background fits. b), c) Background-corrected PELDOR time traces and their fits. d) PELDOR-derived inter-nitroxide distance distributions. Light red and light green shades depict the error estimates for the distance distributions.
Table S6. Relative amounts of functional and twisted TGT dimers (in %).

| Sample          | Functional dimer<sup>a</sup> | Twisted dimer<sup>a</sup> |
|-----------------|------------------------------|---------------------------|
|                 | Tikhonov regularization | DEERNet | Tikhonov regularization | DEERNet |
| TGT(G87R1)      | 100                         | 100         | 0                        | 0               |
| TGT(G87R1)-1, 1 h | 34±6                       | 36±7        | 66±6                     | 64±7           |
| TGT(G87R1)-1, 24 h | 18±6                      | 19±7        | 82±7                     | 81±6           |
| TGT(G87R1)-1, 72 h | 19±6                      | 19±7        | 81±6                     | 81±5           |
| TGT(G87R1)-2, 1 h | 93±6                      | 98±7        | 7±6                      | 2±1            |
| TGT(G87R1)-2, 24 h | 88±6                      | 97±7        | 12±6                     | 3±2            |
| TGT(G87R1)-2, 72 h | 88±10                     | 97±3        | 12±9                     | 3±3            |

<sup>a</sup> The errors were determined in accordance to Section 3.3.
Figure S11. Processing of PELDOR time traces of TGT(G87R1) and TGT(WT)/TGT(G87R1) without and in presence of 1 and 2. All samples were equilibrated for 24 h at room temperature. The results of Tikhonov regularization and DEERNet are shown in red and green, respectively. a) Original PELDOR time traces and their background fits. b), c) Background-corrected PELDOR time traces and their fits. d) PELDOR-derived inter-nitroxide distance distributions. Light red and light green shades depict the error estimates for the distance distributions.
Table S7. PELDOR modulations depth parameters.

| Sample                          | Tikhonov regularization | DEERNet |
|--------------------------------|-------------------------|---------|
| TGT(G87R1)                     | 0.35 ±0.04              | 0.34 ±0.01 |
| TGT(WT)/TGT(G87R1)             | 0.27 ±0.05              | 0.25 ±0.00 |
| TGT(G87R1)-I                   | 0.31 ±0.01              | 0.31 ±0.01 |
| TGT(WT)/TGT(G87R1)-I           | 0.13 ±0.01              | 0.14 ±0.00 |
| TGT(G87R1)-2                   | 0.35 ±0.01              | 0.30 ±0.01 |
| TGT(WT)/TGT(G87R1)-2           | 0.21 ±0.03              | 0.17 ±0.00 |

a The errors were determined in accordance to Section 3.4.
6 PELDOR measurements on the mixture TGT(G87R1)/TGT(H319R1)

Figure S12. Processing of PELDOR time traces of TGT(G87R1)/TGT(H319R1) without and in presence of 1. Both samples were equilibrated for 24 h at room temperature. The results of Tikhonov regularization and DEERNet are shown in red and green, respectively. a) Original PELDOR time traces and their background fits. b), c) Background-corrected PELDOR time traces and their fits. d) PELDOR-derived inter-nitroxide distance distributions. Light red and light green shades depict the error estimates for the distance distributions.

Figure S13. MMM- and MtslWizard-based predictions of the inter-nitroxide distances G87R1-G87R1 (red line), H319R1-H319R1 (green line), and G87R1-H319R1 (blue line) in twisted dimers of TGT(G87R1)-1, TGT(H319R1)-1, and TGT(G87R1)/TGT(H319R1)-1. The MtslWizard models of the spin-labeled mutants were computed using the crystal structure of TGT-1 (PDB-ID 5LPT).
References

[1] S. Jakobi, T. X. P. Nguyen, F. Debaene, S. Cianférani, K. Reuter, G. Klebe, ACS Chem. Biol. 2015, 10, 1897–1907.
[2] A. W. Curnow, F. L. Kung, K. A. Koch, G. A. Garcia, Biochemistry 1993, 32, 5239–5246.
[3] I. Biela, N. Tidten-Luksch, F. Immekus, S. Glinca, T. X. P. Nguyen, H.-D. Gerber, A. Heine, G. Klebe, K. Reuter, PLOS One 2013, 8, e64240.
[4] A. Nguyen, D. Nguyen, T. X. P. Nguyen, M. Sebastiani, S. Dörr, O. Hernandez-Alba, F. Debaene, S. Cianférani, A. Heine, G. Klebe, K. Reuter, ACS Chem. Biol. 2020, 15, 3021–3029.
[5] W. Kabsch, Acta Cryst. D 2010, 66, 125–132.
[6] M. Krug, M. S. Weiss, U. Heinemann, U. Mueller, J. Appl. Crystallogr. 2012, 45, 568–572.
[7] A. J. McCoy, R. W. Grosse-Kunstleve, P. D. Adams, M. D. Winn, L. C. Storoni, R. J. Read, J. Appl. Crystallogr. 2007, 40, 658–674.
[8] E. Potterton, P. Briggs, M. Turkenburg, E. Dodson, Acta Cryst. D 2003, 59, 1131–1137.
[9] P. Emsley, B. Lohkamp, W. G. Scott, K. Cowtan, Acta Cryst. D 2010, 66, 486–501.
[10] P. D. Adams, P. V. Afonine, G. Bunkóczi, V. B. Chen, I. W. Davis, N. Echols, J. J. Headd, L.-W. Hung, G. J. Kapral, R. W. Grosse-Kunstleve, A. J. McCoy, N. W. Moriarty, R. Oeffner, R. J. Read, D. C. Richardson, J. S. Richardson, T. C. Terwilliger, P. H. Zwart, Acta Cryst. D 2010, 66, 213–221.
[11] K. Diederichs, P. A. Karplus, Nat. Struct. Biol. 1997, 4, 269–275.
[12] R. A. Laskowski, M. W. MacArthur, D. S. Moss, J. M. Thornton, J. Appl. Crystallogr. 1993, 26, 283–291.
[13] G. J. Kleywegt, J.-Y. Zou, M. Kjeldgaard, T. A. Jones, Chapter 17.1 Around O in International Tables for Crystallography, International Union of Crystallography, Chester, England, 2006, pp. 353–356.
[14] G. Jeschke, V. Chechik, P. Ionita, A. Godt, H. Zimmermann, J. Banham, C. R. Timmel, D. Hilger, H. Jung, Appl. Magn. Reson. 2006, 30, 473–498.
[15] S. G. Worswick, J. A. Spencer, G. Jeschke, I. Kuprov, Sci. Adv. 2018, 4, eaaat5218.