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1 **Dissecting the open-close transition of a sialic acid TRAP transporter substrate binding**
2 **domain with PELDOR spectroscopy reveals two defined conformational states in solu-**
3 **tion.**

4

5 J. Glaenger, M. Peter, G. H. Thomas, G. Hagelueken

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8

1 **Abstract**

2 The tripartite ATP-independent periplasmic (TRAP) transporters are a widespread class of
3 membrane transporters in bacteria and archaea. Typical substrates for TRAP transporters are
4 organic acids including the sialic acid *N*-acetylneuramic acid (Neu5Ac). The substrate binding
5 proteins (SBP) of TRAP transporters are the best studied component and are responsible for
6 initial high-affinity substrate binding. To better understand the dynamics of the ligand binding
7 process, PELDOR (also known as DEER) spectroscopy was applied to study the conforma-
8 tional changes in the Neu5Ac-specific SBP VcSiaP. The protein is the SBP of VcSiaPQM, a
9 sialic acid TRAP transporter from *Vibrio cholerae*. Spin-labelled double-cysteine mutants of
10 VcSiaP were analysed in the substrate-bound and –free state and the measured distances were
11 compared to available crystal structures. The data were compatible with two clear states only,
12 which are consistent with open and closed forms seen in TRAP SBPs. Substrate titration ex-
13 periments demonstrated the transition of the population from one state to the other with no
14 other observed forms. Mutants of key residues involved in ligand binding and/or proposed to
15 be involved in domain closure were produced and the corresponding PELDOR experiments
16 reveal new insights into the open-closed transition. The results are in excellent agreement
17 with previous *in vivo* sialylation experiments. The structure of the spin labelled
18 Q54R1/L173R1 R125A mutant was solved at 2.1 Å resolution demonstrating no significant
19 changes in the protein structure, suggesting the loss of domain closure is solely due to loss of
20 binding. In conclusion, these data are consistent with TRAP SBPs undergoing a simple two-
21 state transition from an open-unliganded to close liganded state during the transport cycle.
22

1 **Introduction**

2 All bacteria enclose themselves from their environment with at least one membrane. To sur-
3 vive in a given environment, they use membrane transporters to actively import any available
4 nutrients. Although bacteria possess a large variety of substrate specific active transporters,
5 they can be grouped into a small number of major classes: ABC transporters (1), secondary
6 active transporters (2), the phosphotransferase system (PTS) (3) and tripartite ATP-
7 independent periplasmic (TRAP) transporters (4). TRAP transporters are currently the least
8 well studied class. They are absent in eukaryotic organisms but widespread in bacteria and are
9 also found in archaea. A typical TRAP transporter consist of three structural domains: a high
10 affinity substrate binding protein (SBP) and two trans-membrane domains (TMDs) with four
11 and twelve predicted trans-membrane helices (4). The domains are commonly referred to as
12 P-domain (substrate binding protein), Q-domain (smaller TMD) and M-domain (larger TMD).
13 The Q and M domains are either fused into one protein or are expressed as separate proteins
14 that form a tight complex (4). As indicated by their name, TRAP transporters are independent
15 of ATP hydrolysis and some representatives have been shown to rely on a Na⁺ gradient and
16 membrane potential to power the transport mechanism (5, 6). This is considered a reason why
17 TRAPs are especially widespread in marine microorganisms (7). Molecules known to be
18 transported by TRAP transporters range from small organic acids including C4-
19 dicarboxylates, larger sugar acids like *N*-acetylneuramic acid (Neu5Ac) to amino acids (4, 8).
20 Most TRAP transporter substrates contain a carboxylic acid group, which is specifically rec-
21 ognised by the P-domain of the transporter (9).

22 High-resolution structural information about TRAP transporters is currently only available for
23 the soluble P-domains. The first crystal structure of such a domain was solved in 2006 (10)
24 and several more structures either with or without substrate followed (reviewed in (11)). All
25 P-domain structures can be characterized by two $\alpha\beta$ -domains that are connected by an extend-
26 ed hinge helix and a substrate-binding cleft between the two $\alpha\beta$ -domains (Figure 1). In the
27 substrate-bound state, the two $\alpha\beta$ -domains close around the substrate reminiscent of a Venus
28 flytrap and the reverse motion is thought to occur when the substrate is channelled into the
29 transporter (4), likely by an allosteric mechanism through conformational changes in the
30 membrane domains (12). The overwhelming majority of P-domains have a conserved arginine
31 in the substrate binding cleft (position 147 in HiSiaP). This residue is crucial for the substrate
32 interaction by recognizing the afore mentioned carboxylic acid group in the substrate. It
33 thereby acts as a selectivity filter for the transporter, allowing the SBP to recognise organic
34 acids with high affinity and specificity (9). Thus, P-domains are structurally well character-

1 ised in their two “resting states”, namely “open ligand-free” and “close ligand-bound”, and
2 the interactions between substrate and protein are well studied. However, as with all dynamic
3 systems, it is of high interest to analyse how well the crystal structures reflect the solution
4 state. An important question with implications for the mechanism of the whole transporter is,
5 whether in solution, the P-domain is present in equilibrium between open- and closed form or
6 if the conformational change is strictly substrate induced. Also, it is possible that there are
7 additional stable intermediate states of the protein that have not yet been discovered by crys-
8 tallography. Here, pulsed electron-electron double resonance (PELDOR) spectroscopy (also
9 known as double electron-electron resonance (DEER) spectroscopy) was applied to analyse
10 the structure of the P-domain of VcSiaPQM, a Neu5Ac transporter from *Vibrio cholerae*, in
11 solution (13, 14). Site-directed spin labelling (15, 16) was used to introduce nitroxide spin
12 labels at positions that allow to readily distinguish the open- and closed states of the protein.
13 These labelled forms were then used to study the structure of the protein in solution. Further,
14 residues that have been proposed as crucial for the function of the P-domain have been mutat-
15 ed and the effects were analysed. The crystal structure of one of these spin labelled VcSiaP
16 mutants (R125A) was solved at 2.1 Å. The structure verifies that neither the R125A mutation
17 nor the spin labelling process disturbed the overall structure of the protein and it validates the
18 PELDOR distances. Taken together, the results demonstrate for the first time that a TRAP
19 SBP has two clear states in solution, an open unliganded- and close ligand-bound form. This
20 supports current models of an allosteric mechanism for ligand release that is catalysed by con-
21 formational changes in the membrane domains.
22

1 **Materials & Methods**

2 *Cloning, protein expression, purification and spin labelling*

3 The VcSiaP encoding gene (omitting the N-terminal signal sequence) was PCR-amplified
4 from genomic *Vibrio cholerae* DNA using oligos: 5'-GTT ATT CCA TGG GGG CGA CGA
5 CTT TAA AGA TGG GG-3' (forward) and 5-TTC TTC GTC GAC TTA CAT TGC TGC
6 CAA TTT CGA CAC AAT CGG-3' (reverse). The PCR product was cloned into the
7 pBADHisTEV vector (Huanting Liu, University of St Andrews) via the NcoI and Sall re-
8 striction sites. For protein production, the plasmid was transformed into *E. coli* C43 cells. M9
9 minimal media supplemented with 5 % glycerol, 100 µg/ml ampicillin, 2 mM MgSO₄ and
10 0.1 mM CaCl₂ was used to avoid co-purification of Neu5Ac from the medium (17). First, an
11 overnight culture was prepared in LB-media. On the next day a second culture was prepared
12 and inoculated with the overnight culture. The cells were grown to an OD₆₀₀ of 5.0-6.0. The
13 cells were then washed twice by centrifuging at 4000 g for 15 min and resuspending in M9
14 media. 6 l of M9 media were inoculated with 5 ml of the cells (OD₆₀₀ = 5.0-6.0) and incubat-
15 ed at 37 °C for 14-16 h with shaking until an OD₆₀₀ of 0.6 was reached. Each culture was then
16 induced with 500 mg/l L(+)-arabinose and grown for further 5 h at 25 °C. The cells were har-
17 vested by centrifugation at 4000 rpm for 20 min and then flash frozen in liquid nitrogen for
18 storage.

19

20 For purification the cell pellet was resuspended in buffer containing 50 mM Tris-Cl, pH 8,
21 50 mM NaCl and 10 % glycerol (buffer A). A cell disrupter (Constant Systems) was used to
22 lyse the cells twice at 30 kpsi, followed by centrifugation of the lysate at 20.000 rpm for
23 20 min. The obtained supernatant was incubated for 1 h with Ni²⁺ NTA resin (GE
24 Healthcare), which was previously equilibrated with buffer A. The resin was washed with
25 50 ml of buffer A, then with 25 ml of buffer A supplemented with 1 mM Tris(2-
26 carboxyethyl)phosphine (TCEP) to reduce the cysteines of the protein. After another washing
27 step with buffer A to remove the TCEP, the protein was labelled and eluted in one step with
28 15 ml of buffer A containing 31 µL of S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-
29 3-yl)methyl methanesulfonylthioate (MTSSL) and 200 mM imidazole. The sample was then
30 loaded onto a ENrich Q 10/100 column with buffer A and eluted with a linear gradient from
31 0.05-1 M NaCl. Finally, the protein was subjected to size exclusion chromatography on an
32 equilibrated Superdex 75 16/60 or a Superdex 200 16/60 with buffer A as running buffer.

33

34

1 *Continuous wave X-band EPR spectroscopy*

2 The doubly spin labeled proteins were concentrated to 50 μM . EPR spectra were recorded on
3 an EMXnano X-band EPR spectrometer from Bruker. The samples were measured at room
4 temperature with a microwave power of 2.51 mW, a video amplifier gain of 30 dB, a modula-
5 tion amplitude of 1 G, a time constant of 20.48 ms, a conversion time of 21.33 ms and a reso-
6 lution of 10 points per G.

7

8 *PELDOR spectroscopy*

9 For PELDOR spectroscopy, the doubly spin labelled VcSiaP samples (25 μM) were dissolved
10 in PELDOR buffer (100 mM TES pH 7.5, 100 mM NaCl). If needed, the samples were sup-
11 plemented with Neu5Ac and incubated for 30 min on ice. The samples were transferred to a
12 3 mm quartz Q-band EPR tube and flash cooled in liquid nitrogen. The PELDOR time traces
13 were recorded on a Bruker ELEXSYS E580 pulsed Q-band EPR spectrometer, with a ER
14 5106QT-2 Q-band resonator. The instrument was equipped with a continuous flow helium
15 cryostat (CF935) and temperature control system (ITC 502), both from Oxford instruments.
16 The second microwave frequency was coupled into the microwave bridge using a commer-
17 cially available setup from Bruker. All pulses were amplified via a 150 W pulsed travelling
18 wave tube (TWT) amplifier. PELDOR experiments were performed with the pulse sequence
19 $\pi/2(v_A)-\tau_1-\pi(v_A)-(\tau_1+t)-\pi(v_B)-(\tau_2-t)-\pi(v_A)-\tau_2$ -echo. The detection pulses (v_A) were set to 12 ns
20 for the $\pi/2$ and 24 ns for the π pulses and applied at a frequency 80 MHz lower than the reso-
21 nance frequency of the resonator. The pulse amplitudes were chosen to optimize the refocused
22 echo. The $\pi/2$ -pulse was phase-cycled to eliminate receiver offsets. The pump pulse (v_B) was
23 set at the resonance frequency of the resonator and its optimal length (typically 16 ns) was
24 determined using a transient nutation experiment for each sample. The field was adjusted such
25 that the pump pulse is applied to the maximum of the nitroxide spectrum. The pulse amplitude
26 was optimized to maximize the inversion of a Hahn-echo at the pump frequency. All
27 PELDOR spectra were recorded at 50 K with an experiment repetition time of 1 ms, a video
28 amplifier bandwidth of 20 MHz and an amplifier gain of 42 dB. τ_1 was set to 260 ns and the
29 maximum of τ_2 was set to values ranging from 4-12 μs . Deuterium modulation was sup-
30 pressed by addition of 8 spectra of variable τ_1 with a $\Delta\tau_1$ of 16 ns. The obtained time traces
31 were divided by a mono-exponential decay to eliminate intermolecular contributions and
32 renormalized. Distance distributions were obtained from the background corrected data by
33 using the program DeerAnalysis2016 developed by Gunnar Jeschke (18) (The uncorrected

1 time traces are shown in Supporting Figure 1). The influence of different starting points for
2 the background fitting was analysed with the evaluation feature of DeerAnalysis. Linear com-
3 bination fitting of time traces and integration of distance distributions were performed with
4 python (www.python.org) scripts using numpy (www.numpy.org) and scipy (www.scipy.org)
5 functions. The PyMOL (www.pymol.org) plugin mtsslWizard (19) and MMM
6 (<http://www.epr.ethz.ch/software.html>) were used to predict distance distributions.

7

8 *Crystallography*

9 Purified VcSiaP R125A Q54R1/L173R1 at ~17 mg/ml was used to setup crystallisation trials
10 with the JCSG+ Screen (Molecular Dimensions) and 96 well MRC plates (Molecular Dimen-
11 sions). For each drop, 0.5 µl of protein was mixed with 0.5 µl of reservoir solution. A single
12 crystal was observed in condition D7. The crystal was allowed to grow for several weeks at
13 room temperature before harvesting. Prior to flash cooling in liquid nitrogen, the crystal was
14 cryo-protected with 35 % glycerol. Data were collected at beamline BL14.3 of BESSYII
15 (Berlin, Germany), using a MarMOSAIC 225 CCD detector. The data were processed using
16 XDS (20) as implemented in XDSAPP (21). Data collection and processing statistics are
17 listed in Table 1. The structure of VcSiaP was solved using PHASER (22) and PDB-ID
18 4MAG (23) as search model. The PHENIX suite (24) and COOT (25) were used to refine the
19 structure. The geometry of the model was optimised and validated using MOLPROBITY
20 (26).

21

22

1 **Results**

2 *Selection of labelling sites for PELDOR spectroscopy*

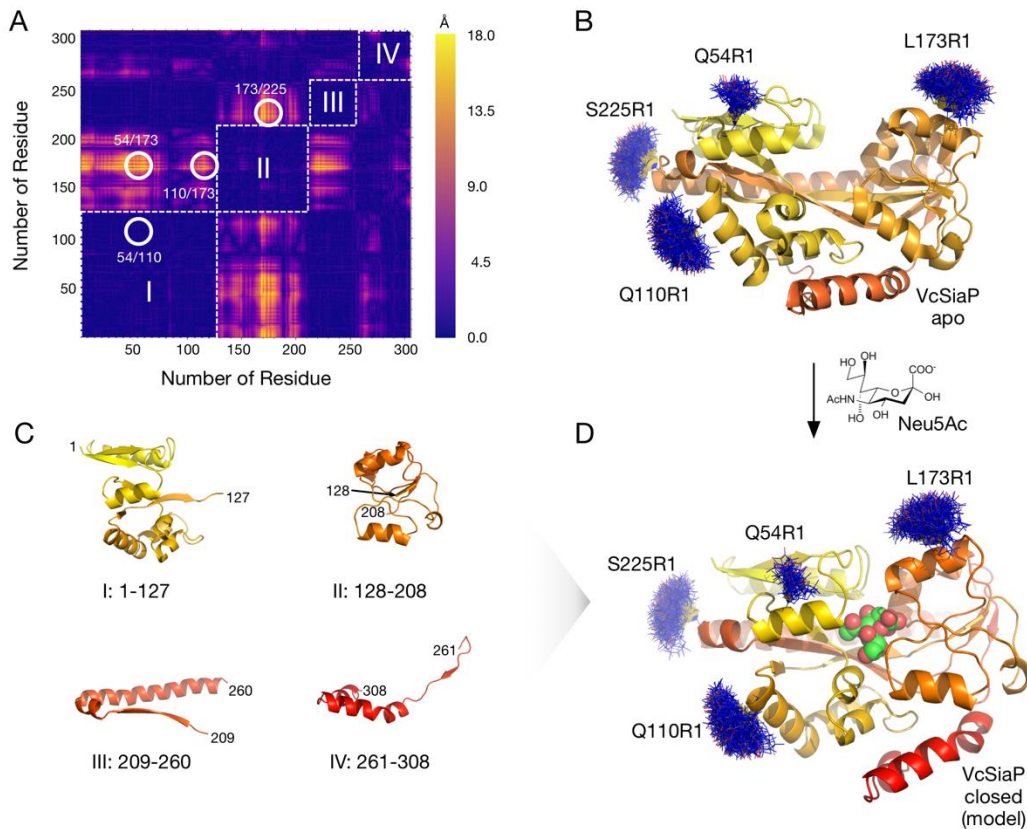
3 To investigate the structures of substrate-bound and -free VcSiaP in solution, PELDOR spec-
4 troscopy was applied (13, 14). This electron paramagnetic resonance (EPR) technique can
5 accurately measure distances between paramagnetic centers in a range of 15 up to 170 Å (27,
6 28) and has frequently been applied to study conformational changes in transporters and
7 channels (29-33). Like most proteins, VcSiaP is diamagnetic, and therefore invisible for EPR.
8 Thus, site-directed spin labelling was used to attach two spin labels to its molecular surface
9 (15). To find optimal labelling positions for VcSiaP, a difference distance matrix (diffDM)
10 between the substrate-bound and -free crystal structures of the homolog HiSiaP (50 % identi-
11 cal amino acids) was calculated (Figure 1A) (34, 35). HiSiaP was used, because no structure
12 of substrate-bound VcSiaP is currently available. The residue numbering between HiSiaP and
13 VcSiaP differs by one or two amino acids (depending on the position in the sequence; see se-
14 quence alignment in Supporting Figure 2). In the following, the VcSiaP numbering is used.
15 The diffDM reveals the absolute value of the spatial displacement between substrate-bound
16 and -free HiSiaP for each possible pair of C β atoms. Consequentially, the distinct yellow
17 peaks in the diffDM (Figure 1A) represent pairs of residues, where the conformational chang-
18 es between the two crystal structures are especially large (up to 18.0 Å). Based on this analy-
19 sis, we selected the residue pairs Q54/L173, Q110/L173, L173/S225 and Q54/Q110 (control)
20 as labelling sites (Figure 1A). The corresponding double cysteine mutants were cloned, ex-
21 pressed and labelled with the MTSSL spin label (36), creating the VcSiaP mutants
22 Q54R1/L173R1, Q110R1/L173R1, L173R1/S225R1 and Q54R1/Q110R1. Judged by room
23 temperature cw-X-band EPR spectroscopy an average labelling efficiency of 90 % was
24 achieved.

25

26 *Building a model of substrate-bound VcSiaP*

27 A diffDM can also be used to identify rigid subdomains within protein structures (35). Be-
28 cause rigid subdomains do by definition not change their conformation between two different
29 states of the protein, they show up as unicolored squares along the diagonal of the diffDM.
30 Here, four such squares were identified (Figure 1A, I-IV). Note that it would be possible to
31 subdivide the squares, if a more fine-grained model was needed and if the coordinate error of
32 the underlying structures was sufficiently small (35). To build a model of substrate-bound
33 VcSiaP, the open structure (PDB-ID: 4MAG (23), Figure 1B) was split at the positions indi-
34 cated by the diffDM to create the rigid subdomains I-IV (I: 1-127, II: 128-208, III: 209-260,

1 IV: 261-308) (Figure 1C). The border between III and IV is close to the “kink” in helix α 9,
 2 which has previously identified as a hallmark of closed P-domains (10). These rigid subdo-
 3 mains of VcSiaP were then superimposed onto the substrate-bound HiSiaP crystal structure
 4 (PDB-ID: 3B50 (37)), leading to a coarse model of substrate-bound VcSiaP. The geometry of
 5 the “cleavage sites” was regularised in COOT (25) (Figure 1D).
 6



7
 8 **Figure 1: Structural changes of P-domains.** **A)** A difference distance matrix (diffDM) for the substrate-bound
 9 and -free forms of HiSiaP (PDB: 3B50 (37), 2CEY (10)). Dark violet regions correspond to pairs of residues,
 10 which do not change their C β -C β distance between both conformations. Yellow peaks indicate large distance
 11 changes of up to 18 Å. White circles mark pairs of residues that were selected as spin labelling sites. The violet
 12 squares along the diagonal of the matrix can be interpreted as rigid domains (I-IV) of the P-domain. Note that the
 13 matrix is symmetric along its diagonal. **B)** The substrate-free structure of VcSiaP (PDB-ID: 4MAG, (23)). The
 14 protein is shown as cartoon model. A color gradient is running from yellow (N-terminus) to red (C-terminus) to
 15 indicate the trace of the polypeptide chain. Models of spin labels highlighted in A) were attached with mtsslWiz-
 16 ard (blue lines). **C)** Cartoon models of the individual structures of the rigid domains of substrate-free VcSiaP. **D)**
 17 Model of the closed form of VcSiaP. The model was produced by superposing the rigid domains in C) onto the
 18 structure of closed HiSiaP (PDB-ID:3B50, (37)). The model of the bound Neu5Ac is shown as spheres.
 19

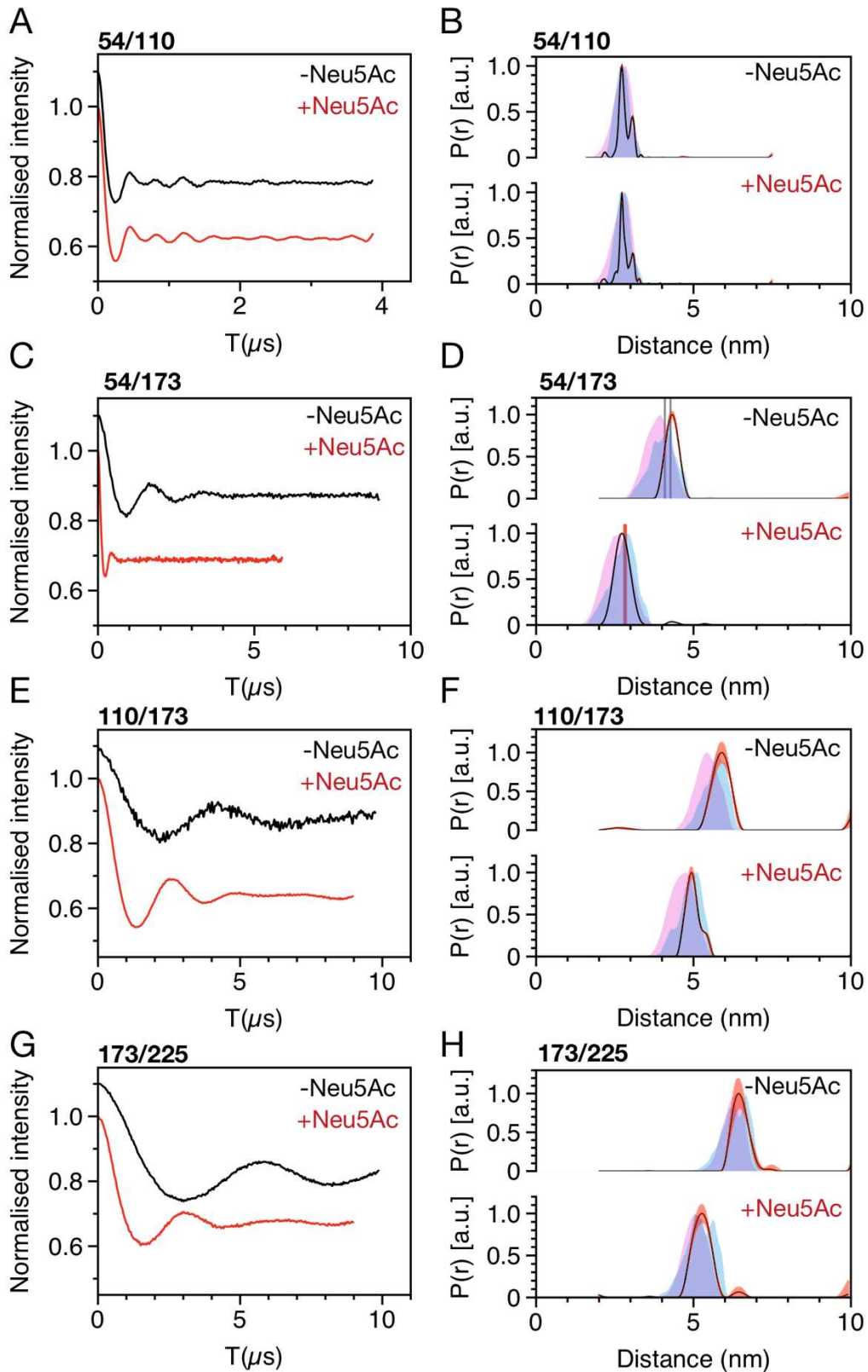
20 Comparing the solution and crystal structures of VcSiaP with PELDOR spectroscopy

21 PELDOR experiments on the doubly spin labelled VcSiaP mutants were conducted. Figure
 22 2A shows the Q-band PELDOR time traces of the “control” mutant, VcSiaP Q54R1/Q110R1,
 23 which according to the crystal structures, should lead to the same distance in the substrate-
 24 bound- and -free state (in Figure 1A, both residues, Q54 and Q110, are located in the same

1 rigid body, “I”). After the initial decay, both time traces show several clear oscillations, indi-
2 cating narrow underlying spin-spin distance distributions. Indeed, as expected for the control
3 sample, the two time traces (\pm Neu5Ac) were virtually identical (Figure 2A). Both time traces
4 were analysed with the DeerAnalysis2016 software (18), leading to the distance distributions
5 in Figure 2B. For both samples, a narrow peak at 27 Å with a shoulder at 30 Å was observed.
6 Models of the open and closed structure with the R1 side chain at positions Q54 and Q110
7 were produced with mtsslWizard (blue sticks in Figure 1BD) and theoretical distance distribu-
8 tions were calculated with mtsslWizard and MMM (shaded areas in Figure 2B) (19). The ex-
9 perimental and expected distributions for VcSiaP Q54R1/Q110R1 agree very well for both
10 experiments (with and without Neu5Ac). A possible explanation for the shoulder at 30 Å is a
11 second conformation of the R1 spin label, which has been frequently observed in available
12 crystal structures of the R1 side chain (38-40).

13 The same procedure was applied to the VcSiaP Q54R1/L173R1 mutant. Again, high quality
14 time traces with clearly visible oscillations were observed (Figure 2C). But, in this case, the
15 PELDOR time traces of the two samples (\pm Neu5Ac) differed strongly. Accordingly, the two
16 corresponding distance distributions show different but well-defined peaks at 27 Å
17 (+ Neu5Ac) or 43 Å (- Neu5Ac) (Figure 2D). Also for this mutant, the experimental distribu-
18 tions show a good match to the predictions made with mtsslWizard and MMM, although for
19 both programs, the predicted distributions are broader than the experimentally determined dis-
20 tributions. The X-ray structure of this mutant revealed that the difference between prediction
21 and experiment is simply due to the prediction error (see below). The experiment was repeat-
22 ed for the VcSiaP Q110R1/L173R1 and L173R1/S225R1 mutants. Also here, clear differ-
23 ences between \pm Neu5Ac were found. Again, the observed distances fit to the mtsslWizard
24 and MMM predictions (Figure 2EF, 2GH). Interestingly, for all double mutants, the room-
25 temperature *cw*-X-band EPR spectra are virtually identical for the apo- or Neu5Ac-bound
26 state, in spite of the large changes of the distance distributions. This indicates that the mobili-
27 ty and possibly also the conformation of the R1 labels does not significantly change between
28 the two states (Supporting Figure 3).

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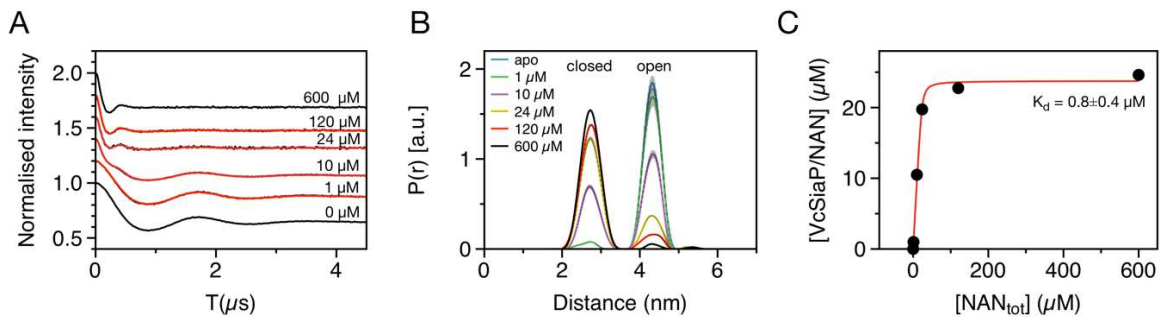
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Figure 2: PELDOR measurements on spin labelled VcSiaP. **A, C, E, G**) Background corrected PELDOR time traces of the indicated VcSiaP double mutant with (red) and without (black) Neu5Ac. **B, D, F, H**) Distance distributions (solid lines) calculated from time traces on the left using DeerAnalysis2016. Predicted distance distributions (mtsslWizard: pink, MMM: light blue) are shown as shades. The error bars (red) were calculated with the “Evaluation” feature of DeerAnalysis2016. Distances from the crystal structure of the spin labelled mutant are shown as vertical lines in D).

1
2 *Following Neu5Ac binding to VcSiaP with PELDOR spectroscopy*

3 To analyse if any stable intermediate states of the P-domain exist, the binding of Neu5Ac to
4 VcSiaP was quantitatively analysed by PELDOR spectroscopy. For this purpose, samples of
5 VcSiaP Q54R1/L173R1 (25 μM) supplemented with different amounts of Neu5Ac were pro-
6 duced (0 to 600 μM Neu5Ac). This particular mutant was chosen, because it showed the
7 clearest difference between substrate-bound and -unbound state (Figure 2). A Q-band
8 PELDOR time trace was recorded for each sample (Figure 3A). The corresponding distance
9 distributions were calculated with DeerAnalysis and each normalised to an integral value of
10 1.0 (Figure 3B).

11



12
13 **Figure 3: Open-close transition of VcSiaP followed by PELDOR spectroscopy.** A) PELDOR time traces of
14 VcSiaP Q54R1/L173R1 titrated with the indicated amounts of Neu5Ac. The red curves are fits resulting from
15 linear combinations of the 0 μM (open) and 600 μM (close) Neu5Ac time traces using equation $y = a \cdot \text{open} + (1 -$
16 $a) \cdot \text{close}$. Note that small differences in modulation depths were corrected by scaling the time traces to a modula-
17 tion depth of 100 % prior to the fitting procedure. The fitting results were then back-scaled to the original modu-
18 lation depth. B) Distance distributions corresponding to the time traces shown in A). The distributions were
19 normalised, so that their integral equals 1.0. The error bars (grey) were calculated using the evaluation procedure
20 from DeerAnalysis. C) Binding isotherm of the VcSiaP Q54R1/L173R1 *Neu5Ac interaction. The black dots
21 represent the calculated VcSiaP/Neu5Ac concentrations (see main text). The solid red line represents a fit of the
22 equation $y = ((P_{\text{tot}} + \text{Lig}_{\text{tot}} + K_d) - \sqrt{(P_{\text{tot}} + \text{Lig}_{\text{tot}} + K_d)^2 - 4 \cdot P_{\text{tot}} \cdot \text{Lig}_{\text{tot}}}) / 2$ (41) to the data points. P_{tot} is the total con-
23 centration of VcSiaP, Lig_{tot} is the total amount of Neu5Ac and K_d the dissociation constant. A K_d of
24 $0.8 \pm 0.4 \mu\text{M}$ was determined.

25

26 Assuming that the PELDOR distance distributions quantitatively reflect the state of VcSiaP in
27 solution, the peak area of the substrate-bound (closed) form of each sample (Figure 3B)
28 should be directly proportional to concentration of the VcSiaP/Neu5Ac complex. Vice versa,
29 the peak area of the substrate-free (open) form should be proportional to the VcSiaP_{free} con-
30 centration. The two peaks were therefore integrated for each sample and the integral values
31 converted to concentrations (Supporting Table 1). **Tikhonov regularization as implemented in**
32 **DeerAnalysis2016 was used to extract the distance distributions from the PELDOR time trac-**
33 **es. On principle, the same PELDOR time trace will yield distance distributions of differing**
34 **width and/or shape depending on the choice of the Tikhonov regularization parameter α . Alt-**

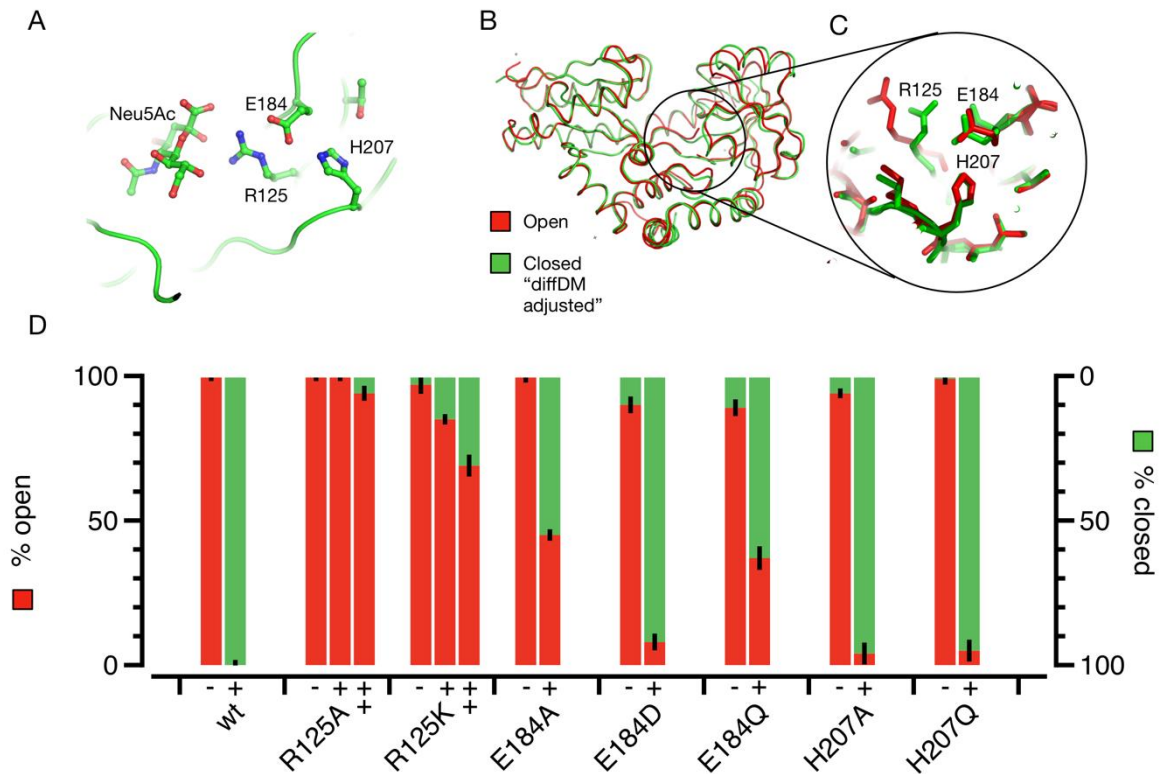
1 hough DeerAnalysis automatically chooses the optimal α -value based on an L-curve criterion,
2 it cannot be excluded that this procedure influenced the integrals that were calculated above.
3 It was therefore tried to extract the fractions/concentrations of apo- and Neu5Ac-bound
4 VcSiaP directly from the background corrected PELDOR time traces (Figure 3A). For this
5 purpose, the time traces from the intermediate (1-120 μ M) Neu5Ac concentrations were fitted
6 as linear combinations of the apo- and fully substrate-bound time traces. The red lines in Fig-
7 ure 3A show that the resulting fits almost perfectly reproduce the experimental data. The re-
8 sulting fractions/concentrations of apo- and Neu5Ac-bound VcSiaP and their estimated uncer-
9 tainties are listed in Supporting Table 1. Reassuringly, the concentrations from both the linear
10 combination and integration methods matched very well (Supporting Table 1). A binding iso-
11 therm was plotted using the mean of the calculated concentrations from both methods. (Figure
12 3C) and a dissociation constant of $0.8 \pm 0.4 \mu$ M was determined by non-linear fitting of equa-
13 tion $y = ((P_{\text{tot}} + \text{Lig}_{\text{tot}} + K_d) - \sqrt{(P_{\text{tot}} + \text{Lig}_{\text{tot}} + K_d)^2 - 4 * P_{\text{tot}} * \text{Lig}_{\text{tot}}}) / 2$ to the data points (41). Note,
14 that for an optimal binding experiment, the concentration of VcSiaP should have been signifi-
15 cantly below the expected K_d value to avoid substrate depletion (41). Here, in order to record
16 PELDOR time traces with good signal to noise ratio, much higher concentrations of 25 μ M
17 VcSiaP were used. The consequence was a very sharp transition in the binding isotherm,
18 which makes it difficult to accurately determine the K_d value. Nevertheless, the obtained K_d
19 value is reasonably close to previously published values (0.3 μ M, (23) and 0.1 μ M (5, 6)),
20 suggesting that the PELDOR distance distributions of VcSiaP 54R1/173R1 can be quantita-
21 tively analysed in the described ways.

22

23 *Mutational analysis of open-close transition*

24 Whereas the role of R147 as a selectivity filter in P-domains is established (9), it is currently
25 not known, how exactly the bound substrate triggers the conformational change of the P-
26 domain. In Neu5Ac binding P-domains, a group of three conserved, polar amino acids, R125,
27 E184 and H207 were observed to form an intricate network of interactions (23). It has been
28 proposed that these residues play an important role in the open-closed transition of P-
29 domains, also because they are close to two “hinge regions” in the structure (Figure 4A) (23).

30



1
2 **Figure 4: Conformational changes upon Neu5Ac binding.** A) Detail of the substrate bound HiSiaP structure
3 (PDB-ID: 3B50, (37)), showing the Neu5Ac molecule and its interaction with R125, E184 and H207 (VcSiaP
4 numbering). B) Superposition of the four rigid bodies of substrate bound (green) HiSiaP (Figure 1) with substrate-free HiSiaP (red, PDB-ID: 2CEY, (10)). C) Detail of the superposition in B), showing the same R125,
5 E184, H207 cluster. D) Open/closed state (percentage) of VcSiaP mutants as determined by PELDOR spectroscopy. The PELDOR data is shown in Supporting Figure 4. The ++/+/- indicates if 10 mM (++)
6 1 mM (+) or no (-) Neu5Ac was present in the experiment. The error bars represent ± 3 -times the standard deviation calculated in
7 the linear combination fitting procedure.
8
9

10
11 To better visualise the conformational changes of these amino acids upon substrate binding,
12 the closed structure of HiSiaP was split into four rigid bodies as indicated by the diffDM
13 (Figure 1) and the rigid bodies were superposed onto the open structure (Figure 4B, r.m.s.d. =
14 0.45 for 308 C α atoms). In this way, the conformational changes of the side chains are not
15 obstructed by the larger scale rigid body movements of the protein backbone and therefore
16 easier to analyse. Indeed, the superposition revealed that the conformation of R125 changes
17 upon substrate binding, whereas H207 and E184 appear unchanged (Figure 4C).

18 The three residues were systematically mutated in the VcSiaP Q54R1/L173R1 construct to
19 analyse their individual influence on Neu5Ac binding. PELDOR measurements in the pres-
20 ence and absence of 1 mM Neu5Ac were conducted with the purified mutants. According to
21 the titration experiment above (Figure 3), 1 mM Neu5Ac suffices to induce the closed state in
22 the “wild-type” protein. The time traces and distance distributions are compiled in Supporting
23 Figure 4. For each experiment, the percentage of open versus closed VcSiaP was determined

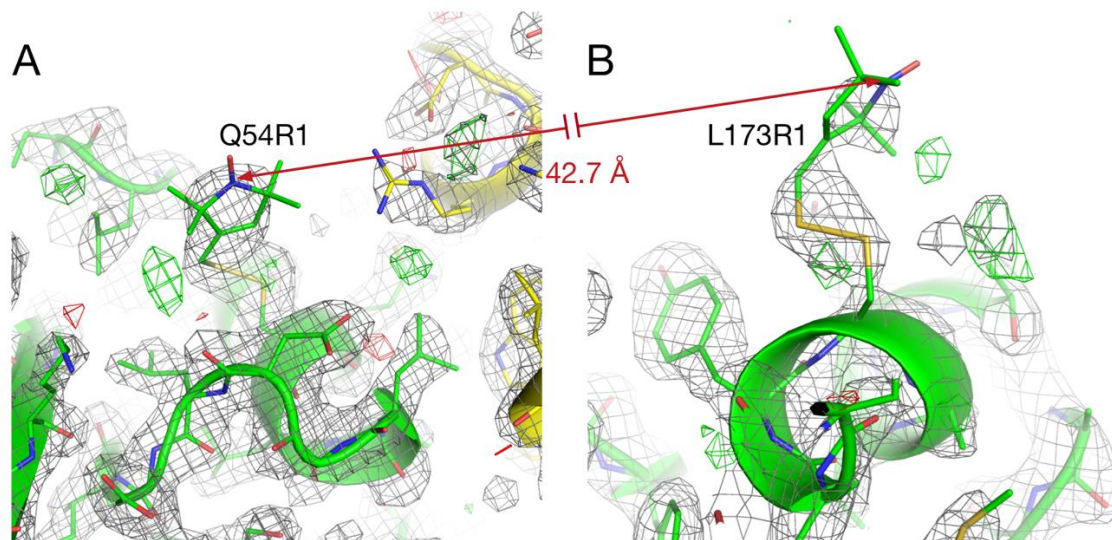
1 by linear combination fitting as described above (Figure 4D, Supporting Figure 4). The error
2 bars in Figure 4D represent the estimated uncertainty of the open/closed fractions (Supporting
3 Table 2). First, R125 was mutated to alanine. The PELDOR experiments reveal that with
4 1 mM Neu5Ac, no significant amount of the substrate-bound (closed) conformation could be
5 detected (Figure 4D). However, at 10 mM Neu5Ac, the amount of closed VcSiaP increased to
6 $6\pm 2\%$. To conserve the positive charge of R125, the residue was also mutated to lysine. In
7 this case, a small but significant percentage ($15\pm 2\%$) of VcSiaP/Neu5Ac complex was ob-
8 served with 1 mM ligand, while most of the protein ($85\pm 2\%$) remained in the open confor-
9 mation (Figure 4D). Also here, increasing the Neu5Ac concentration to 10 mM led to a larger
10 percentage of closed VcSiaP ($31\pm 3\%$). Next, E184 was mutated to alanine. The PELDOR
11 measurement with 1 mM Neu5Ac revealed a $\sim 1:1$ mix of open and closed VcSiaP. In con-
12 trast, the charge conserving E184D mutant behaved almost like the wild-type in our experi-
13 ments (Figure 4D). Interestingly, the charged to polar mutant E184Q behaved similar to the
14 E184A mutant, indicating that the ionic interaction of residue 184 with R125 is important,
15 while small structural changes (E \rightarrow D) can be tolerated (Figure 4D). Finally, H207 was mu-
16 tated to alanine or glutamine. According to Figure 4A, a glutamine at position 207 should still
17 be able to form a polar interaction with E184. Both H207 mutants appeared to be less stable
18 than the wild-type protein. For example, aggregate peaks were observed (and removed) in
19 gelfiltration experiments (Supporting Figure 5). However, once purified, both H207A and
20 H207Q behaved almost like the wild-type protein in the PELDOR experiments (Figure 4D).

21

22 *X-ray structure of spin labelled VcSiaP R125A Q54R1/L173*

23 To check whether the very low binding activity of the R125A Q54R1/L173R1 mutant is
24 caused by a change in its overall structure, the protein was crystallised. Initial crystals were
25 obtained in condition D7 of the JCSG+ screen. The crystals were optimised and a 2.1 Å dif-
26 fraction dataset was collected. The structure was solved by molecular replacement with
27 PHASER (22), using the wild-type structure as search model (PDB-ID: 4MAG, (23)).

28



1
2 **Figure 5: X-ray structure of spin labelled VcSiaP R125A Q54R1/L173R1.** A) The R1 side chain at position
3 54. The protein backbone is shown as a green cartoon model with sticks. A neighbouring molecule in the crystal
4 is coloured yellow. The grey mesh represents 2mFo-DFc electron density contoured at 1.0 σ . The green and red
5 meshes are respectively positive and negative peaks in the mFo-DFc electron density contoured at 3.0 σ . B) The
6 R1 side chain at position 173. The figure is analogue to A). The distance vector between the two spin centers is
7 indicated by a red arrow. Its absolute value is 42.7 Å (The N-N distance was measured).
8

9 The asymmetric unit contained 2 copies of the protein. Refinement with phenix.refine led to
10 R/R_{free} -factors of 22.2/25.9. Molprobity was used to validate the stereochemistry of the model
11 (26). Data collection and refinement statistics are listed in Table 1. The final, refined VcSiaP
12 Q54R1/L173R1 structure and the search model superpose with an rmsd of 0.3 Å for 260 C α
13 atoms for chain A and with an rmsd of 0.5 Å for 282 C α atoms for chain B. Supporting Figure
14 6 shows that chain A fits almost perfectly to the previously published wild-type structure,
15 while chain B is in a slightly more closed conformation. Such slightly closed states of un-
16 liganded P-domains were predicted by MD simulations (12). While both spin labels are clear-
17 ly visible in the electron density, the 54R1 sidechain is better defined and therefore apparently
18 less mobile (Figure 5). This fits to the observation that the conformational ensemble produced
19 by mtsslWizard and MMM is much smaller for Q54R1 than for L173R1 (Figure 1 BD). De-
20 tails about the conformation of the two spin labels including the dihedral angles of the side
21 chains are compiled in Supporting Figure 7. The distance between the Q54R1 and L173R1
22 spin centers was measured for both chains in the crystal structure and amounts to 42.7/40.9 Å.
23 Both distances fit well to the corresponding PELDOR result (Figure 2D, grey lines). The *cw*-
24 X-band EPR spectra of Q54R1/L173R1 \pm Neu5Ac indicated that the mobility of the spin label
25 (and therefore likely also its molecular surrounding) does not significantly change upon
26 Neu5Ac binding (Supporting Figure 3). Thus, as explained above, the crystal structure was
27 separated into rigid-bodies I-IV (Figure 1C) and superimposed onto the closed-state HiSiaP

1 structure. Now the distance between the two spin labels was again measured and fits very well
2 to the measured PELDOR distance of the closed state (Figure 2D, red lines).

3 In summary, neither the R125A mutation nor the attachment of the two spin labels signifi-
4 cantly disturbed the overall structure of VcSiaP. Further, our structure is another indication
5 that crystal structures of the R1 side chain can be good approximations for the rotameric state
6 of the side chain in frozen solution (i.e. in PELDOR samples) (39, 40).

7
8 **Table 1: Data collection & refinement statistics**

	VcSiaP Q54R1/L173R1 (PDB-ID: 5LTC)
Wavelength	0.89429
Resolution range	45.29 - 2.101 (2.176 - 2.101)
Space group	P 2 ₁ 2 2 ₁
Unit cell	72.2697 78.1 116.24 90 90 90
Total reflections	73484 (6631)
Unique reflections	37987 (3606)
Multiplicity	1.9 (1.8)
Completeness (%)	97 (94)
Mean I/sigma(I)	7.25 (1.18)
Wilson B-factor	40.2
R-merge	0.043 (0.51)
R-meas	0.061 (0.73)
CC1/2	0.997 (0.507)
CC*	0.999 (0.82)
Reflections used in refinement	37954 (3605)
Reflections used for R-free	2019 (188)
R-work	0.222 (0.357)
R-free	0.259 (0.359)
CC(work)	0.967 (0.517)
CC(free)	0.957 (0.438)
RMS(bonds)	0.005
RMS(angles)	0.78
Ramachandran favored (%)	98
Ramachandran allowed (%)	1.5
Ramachandran outliers (%)	0
Rotamer outliers (%)	0.78
Clashscore	7.60
PDB-ID	5LTC

9 Values in parentheses correspond to the shell of highest resolution.

10

1 Discussion

2 The PELDOR-based binding study on the VcSiaP Neu5Ac interaction (Figure 3) led to a K_d
3 value, which is close to previously determined values using isothermal titration calorimetry
4 (ITC) or Trp fluorescence quenching (5, 6, 23). This suggests that the PELDOR data can be
5 quantitatively analysed and, judged by the error calculated from the linear combination fitting
6 of VcSiaP Q54R1/L173R1 time traces, it appears that $\geq 3\%$ of the closed conformation can be
7 detected (Supporting Table 2). This knowledge is vital for the discussion of the mutational
8 analysis below. It should be noted, that for mutants that do not produce an equally drastic
9 change of the PELDOR time traces upon addition of Neu5Ac (Figure 2C) and for lower sig-
10 nal to noise ratios, this detection limit will be higher. Such PELDOR-based binding experi-
11 ments are time consuming but offer the opportunity to measure the concentration of both the
12 ligand-bound and -free states of the protein and at the same time to gain information about the
13 structural state of the protein. This is usually not possible with more simple biochemical bind-
14 ing assays. “Nevertheless, it will be difficult to precisely determine low K_d -values ($\ll 1\ \mu\text{M}$)
15 with PELDOR, because it is currently experimentally not feasible to use the necessary nano-
16 molar concentrations of spin labelled protein, while still measuring time traces with suffi-
17 ciently high signal/noise (S/N) ratio. However, it has to be determined for each particular
18 case, which S/N ratio is really needed to accurately distinguish the two states. In contrast, the
19 method might be advantageous for large K_d -values: For the R125A and R125K mutants, no
20 Neu5Ac binding had previously been detected via ITC (23), while the PELDOR experiment
21 indicates weak binding. It should be noted that the PELDOR samples were flash frozen.
22 Therefore, if a K_d can be determined, it might differ from a K_d that was determined at room
23 temperature, depending on the k_{on} and k_{off} rates compared to the time needed for the freezing
24 process. The recently developed trityl spin labels, which can be used at room temperature
25 might be a possibility to avoid this problem (42-44). However, these labels are considerably
26 larger than the MTSSL label and are currently still not used on a routine basis. Förster reso-
27 nance energy transfer spectroscopy (FRET) is another possible alternative and can even de-
28 termine the k_{on} and k_{off} rates of the interaction. However, since FRET labels are usually quite
29 large compared to spin labels such as the R1 side chain, the structural information might be
30 less informative than what can be gained from PELDOR experiments.

31 Several high-resolution crystal structures of TRAP transporter P-domains, both in the sub-
32 strate-bound and -free state have been solved in the last decade (reviewed in (11, 45)),
33 providing a detailed picture of the overall structures of P-domains and their interaction with
34 their particular substrate. The PELDOR results from this study indicate that the crystal struc-

1 tures of Neu5Ac binding P-domains are very good models for the solution state of the pro-
2 teins. The distance distributions that were predicted from the crystal structures using
3 mtsslWizard and MMM fit nicely to the experimental data. An even better fit was obtained
4 for the spin labelled crystal structure (Figure 2), indicating that the small differences between
5 experiment and prediction can be explained by the known error of the prediction algorithms
6 ($\pm 3 \text{ \AA}$, (19, 46, 47)). Within the detection limit of the PELDOR experiments, the P-domains
7 of Neu5Ac TRAP transporters appear to almost exclusively adopt the open state in the ab-
8 sence of ligand. For ABC transporters, the substrate binding proteins (SBP) of the GlnPQ
9 amino acid ABC transporter from *L. lactis* and of the maltose ABC transporter were shown to
10 fluctuate between the closed- and open state even in the absence of ligand (48, 49), while the
11 SBP of a glutamine ABC transporter (GlnBP) appears to remain in the open state without lig-
12 and (50). The data above further demonstrate that within the detection limit of the PELDOR
13 experiments there is no trace of any stable intermediate states of the P-domain in solution
14 (Figure 3). These results are in agreement with MD simulations, where no stable intermediate
15 states were predicted for the P-domain of the ectoine TRAP transporter TeaABC (12). The
16 slightly different open conformations that were present in our crystal structure (Supporting
17 Figure 6) fit to the relatively broad energetic minimum for the open structure that was deter-
18 mined in those calculations (12). Considering the current hypothesis for the transport cycle of
19 TRAP transporters (4), large concentrations of “close ligand-free” P-domains would trigger
20 unproductive closing and opening of the transporter. One might speculate that VcSiaP was
21 evolutionary optimized to only close when the substrate is bound, thereby increasing the effi-
22 ciency of the transporter. It should again be noted that the PELDOR experiments are conduct-
23 ed using flash frozen VcSiaP solutions. It cannot be ruled out that during the freezing process,
24 any transition states or “close ligand-free” molecules have snapped back to the open state and
25 were thus not observed. Also, the PELDOR data only show the steady-state of the sample.
26 Transient states that were indicated by stopped-flow fluorescence spectroscopy analysis of
27 other TRAP SBPs (51-53) might be present at low concentrations ($\leq 3 \%$). “Time-resolved”
28 PELDOR experiments using freeze quench instrumentation (54, 55) might be a possible (but
29 experimentally demanding) way to investigate the structure of such transient states.

30 As mentioned above, the R125-E184-H207 triade (henceforth referred to as “triad”) is located
31 in the hinge I & II regions of Neu5Ac transporter P-domains, implicating a role in triggering
32 the conformational change between substrate-free- (open) and –bound (closed) state of the
33 protein (23). In a previous study, isothermal titration calorimetry was used to determine the
34 Neu5Ac binding characteristics of triad mutants (23). The PELDOR from this work now

1 gives the opportunity to correlate the mere ability to bind Neu5Ac with the ability of the par-
2 ticular mutant to perform an open-close transition. Firstly, all triad mutants were able to adopt
3 the open state with wild-type-like, sharp distance distributions. Also, if the closed state was
4 observed for a particular mutant, the same average distance as for the “wild-type” protein was
5 observed (Supporting Figure 4). This strongly suggests that the triad is not necessary for the
6 P-domain to adopt its native open or closed conformation. The R125A mutant was structural-
7 ly intact (Supporting Figure 4), but at 1 mM Neu5Ac, only a very low percentage of the
8 closed state was observed (Figure 4D). To verify, if this very small fraction of closed state
9 was not an artefact, the PELDOR experiment was repeated with 10 mM Neu5Ac, resulting in
10 4 ± 2 % closed state (Figure 4D). Thus, although the R125A mutant binds Neu5Ac very weak-
11 ly (23), it can still correctly adopt the closed state. The same weak-binding phenotype had al-
12 so been observed for the R125K mutant (23), but, using PELDOR, a small but significant per-
13 centage (15 ± 2 %) of the protein was clearly observed in the closed state (Figure 4D). Also
14 here, increasing the Neu5Ac concentration to 10 mM led to an increase of the closed-state
15 percentage to 36 ± 3 % (Figure 4D). So, similar to R125A, the R125K mutant binds Neu5Ac
16 very weakly, but has clearly not lost its ability to reach the closed state. According to the crys-
17 tal structure of substrate-bound HiSiaP (Figure 4A), R125K should still be able to form an
18 ionic interaction with E184 (2.8 \AA), but its amino group will be too far from the Neu5Ac
19 binding site to strongly interact with the substrate ($> 4.8 \text{ \AA}$). This explains the very weak
20 Neu5Ac binding of both mutants and why R125K binds stronger than R125A. The mutational
21 data on E184 reveal that the residue is important, but not crucial for the function of the P-
22 domain. While the E→A mutant is still 55 ± 1 % closed at 1 mM Neu5Ac, the charge conserv-
23 ing E→D mutant was almost indiscernible from the wild-type protein. Also in this case, the
24 PELDOR data agree well with available binding data (23). E184 thus seems to simply stabi-
25 lize R125 by an ionic interaction, keeping the latter in an optimal state to interact with the
26 substrate. H207 does not seem to play any important role in the substrate-induced closing
27 mechanism of the P-domain, because even the H→A mutant was 96 ± 1 % closed in the pres-
28 ence of 1 mM Neu5Ac. However, as mentioned above, the protein was less stable when this
29 mutation was introduced. This might be the reason for the reduced binding affinity that was
30 previously observed (23). The triad residues have also been mutated in an earlier study on
31 nontypeable *H. influenzae* and the effects of the mutations on LPS sialylation were analysed
32 *in vivo* by complementation assays (37). Strikingly, the *in vivo* effects fit perfectly to the
33 PELDOR results in Figure 4D: R125A showed no sialylation, E184Q and R125K partial si-
34 alylation and H207A full sialylation (residue numbers given in VcSiaP numbering).

1 In summary, the impact of mutating the individual three residues of the R125, E184, H207
2 triade varies strongly. R125 is clearly of high importance, presumably because of its interac-
3 tion with the substrate. However, based on the available data, the network of interactions be-
4 tween the triade sidechains does not seem to act as a “substrate sensor”, which triggers the
5 conformational changes between substrate-free and -bound states of P-domains.

6

7 **Conclusion & Outlook**

8 The solution structure and open-close transition of VcSiaP was analysed with PELDOR spec-
9 troscopy, revealing that the crystal structures of both, the open- and closed states are good
10 models for the solution structure of the P-domain in either state. In the absence of substrate
11 and within the detection limit of the PELDOR experiments, the P domain is exclusively found
12 in the opened state. No indications of stable intermediate states were found in PELDOR-based
13 titration experiments. A mutational analysis of the R125, E184, H207 triade was conducted.
14 R125 is primarily involved in substrate binding and is stabilised by its interaction with E184.
15 H207 does not appear to play a vital role in the open-close transition but mutating this posi-
16 tion leads to a less stable VcSiaP protein. In future experiments, we aim to analyse the struc-
17 ture and function of VcSiaP in the context of the transmembrane domains VcSiaQM.

18

19

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8

1 **Author contributions**

2 JG, MP and GH performed experiments and analysed data. GH and GHT designed experi-
3 ments and wrote the paper. GH conceived the study.

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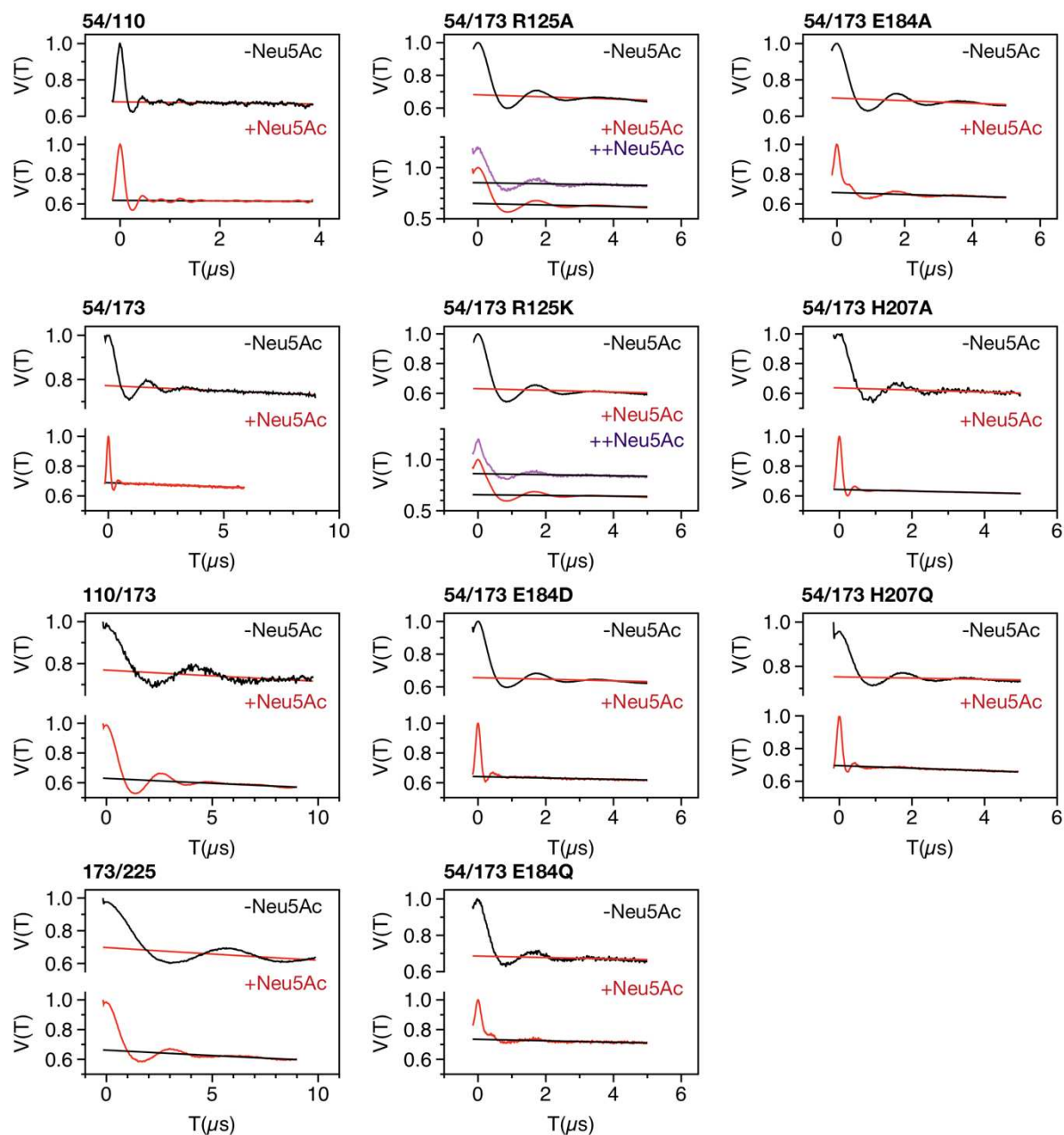
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Supporting Information

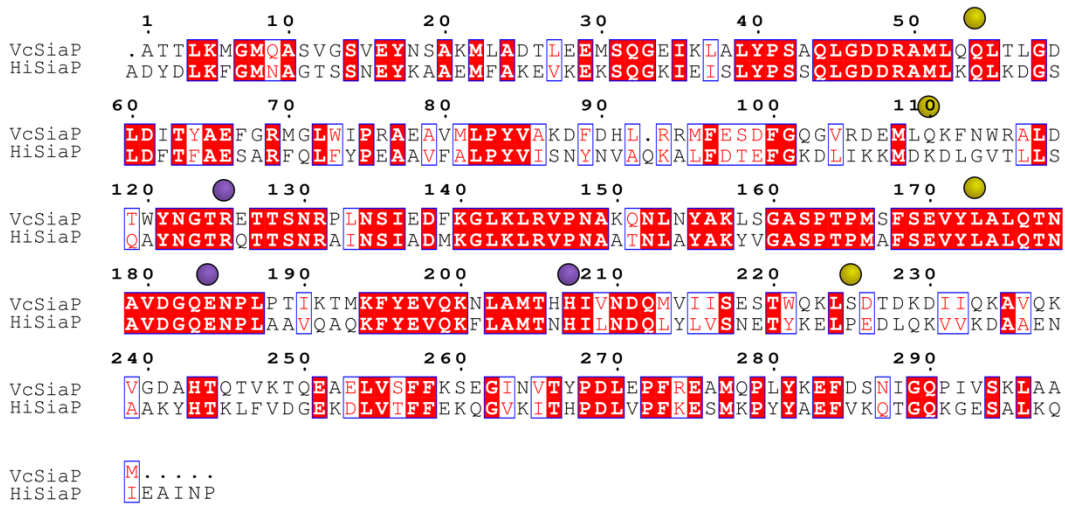
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 3 **Supporting Figure 1:** Uncorrected PELDOR time traces for the indicated mutants in the ab-
 4 sence (-) or presence of 1 mM (+) or 10 mM (++) Neu5Ac. The intermolecular background
 5 that was used for the background correction is indicated. The difference in modulation depths
 6 for H207Q(+/-) are due to different pump pulse positions. For E184Q (+/-), R125A (+/-) and
 7 R125K (+/-) the small differences in modulation depths are due to slightly different pump
 8 pulse lengths (14 vs 16 ns).

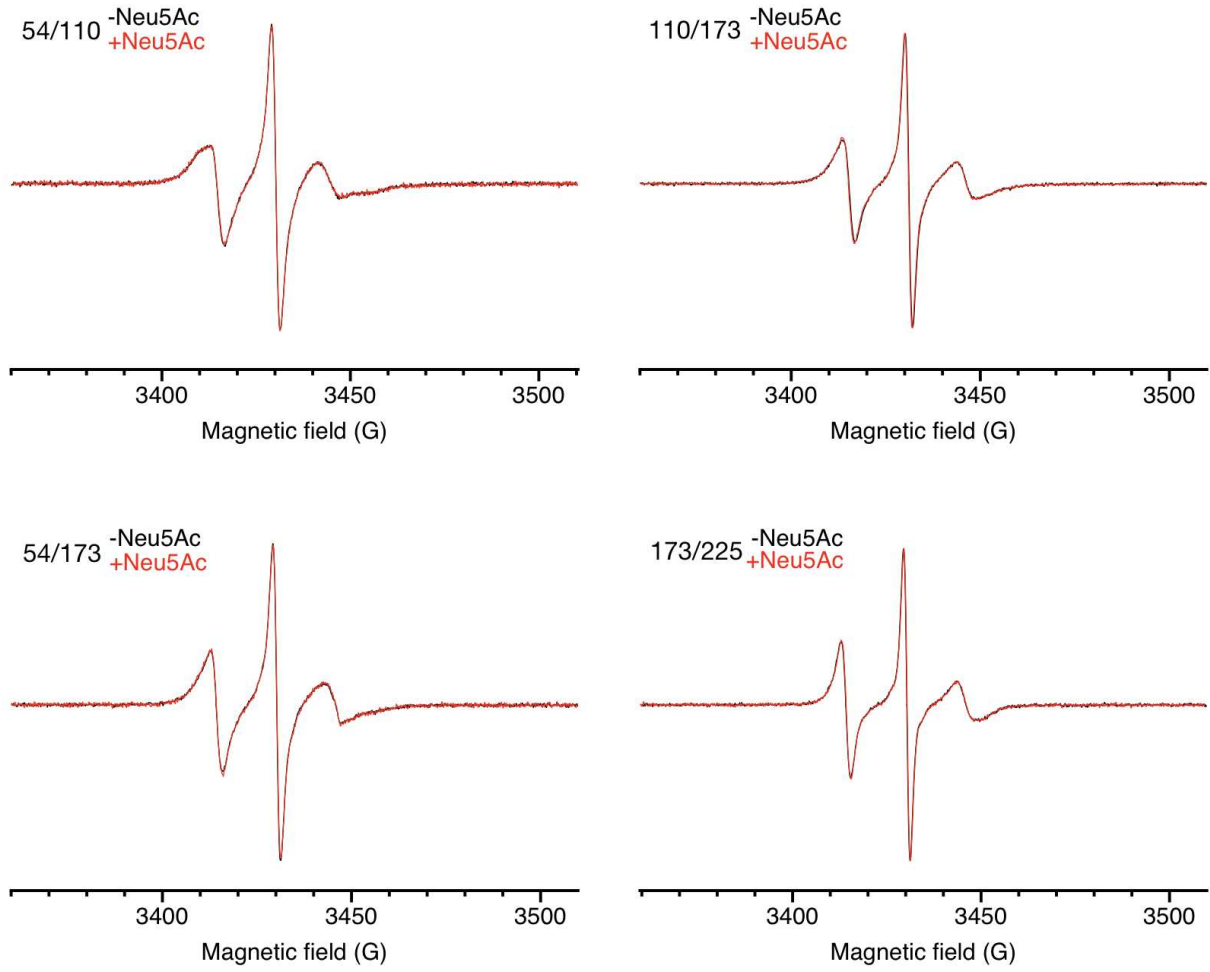
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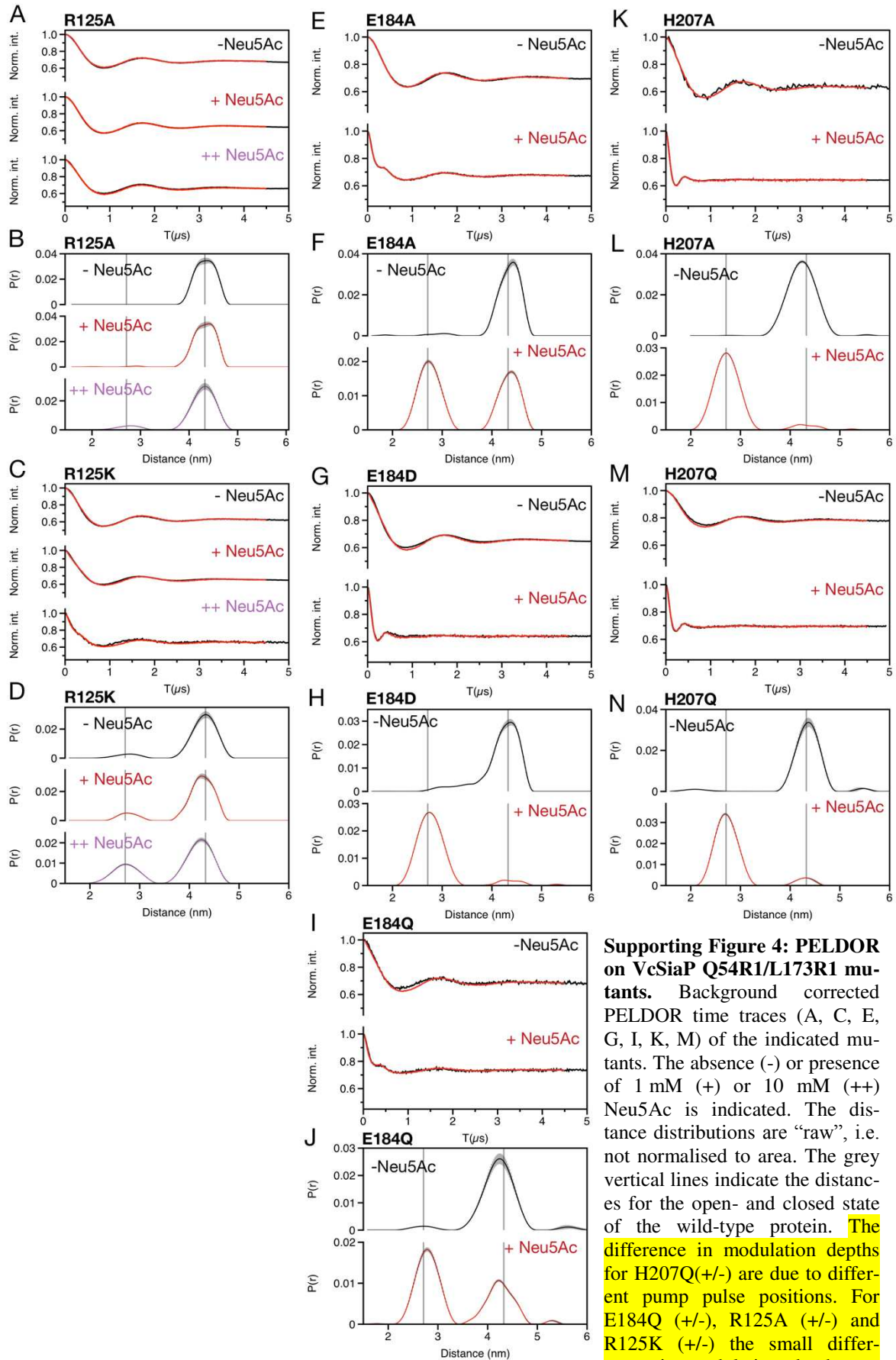
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Supporting Figure 2: Sequence alignment of HiSiaP from *Haemophilus influenzae* and VcSiaP from *Vibrio cholera*. Spin label positions are indicated by yellow spheres. Mutated residues of the conserved triad (R125, E184, H207) are marked by purple spheres.

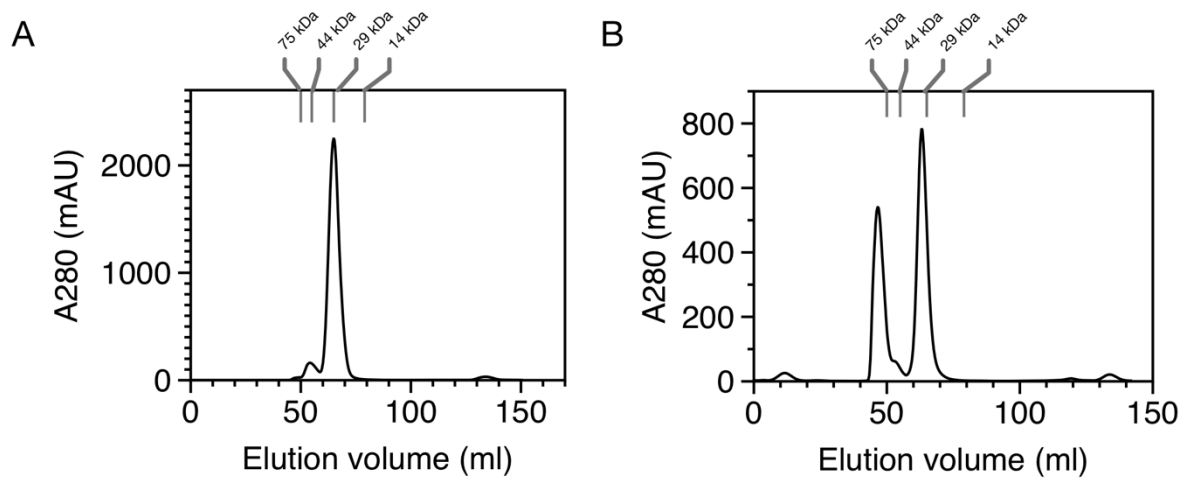


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Supporting Figure 3: Room temperature *cw*-X-band EPR spectra of the indicated VcSiaP double mutants in the absence (-) and presence (+) of 1 mM Neu5Ac.



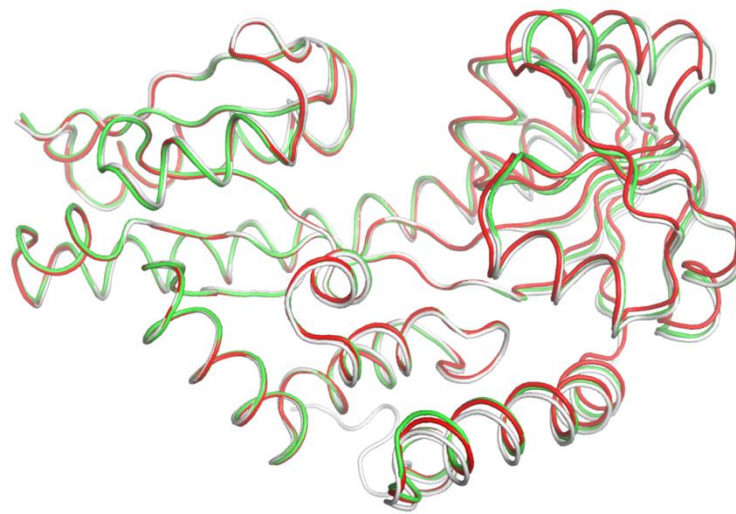
Supporting Figure 4: PELDOR on VcSiaP Q54R1/L173R1 mutants. Background corrected PELDOR time traces (A, C, E, G, I, K, M) of the indicated mutants. The absence (-) or presence of 1 mM (+) or 10 mM (++) Neu5Ac is indicated. The distance distributions are “raw”, i.e. not normalised to area. The grey vertical lines indicate the distances for the open- and closed state of the wild-type protein. **The difference in modulation depths for H207Q(+/-) are due to different pump pulse positions. For E184Q (+/-), R125A (+/-) and R125K (+/-) the small differences in modulation depths are due to slightly different pump pulse lengths (14 vs 16 ns).**



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2 **Supporting Figure 5: Gelfiltration of VcSiaP Q54R1/L173R1 mutants.** A) VcSiaP
3 Q54R1/L173R1 “wt”. A Superdex 75 16/60 column was used. Molecular weight markers are
4 indicated. The protein runs as a monomer, no aggregates were observed. **B)** VcSiaP
5 Q54R1/L173R1 H207Q. A Superdex 75 16/60 column was used. In contrast to all other mu-
6 tants, aggregates were observed for the H207 mutants. The monomer peak at ~65 ml was
7 isolated and used for the PELDOR measurements.

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- VcSiaP R125A Q54R1/L173R1 - chain A
- VcSiaP R125A Q54R1/L173R1 - chain B
- VcSiaP 4MAG

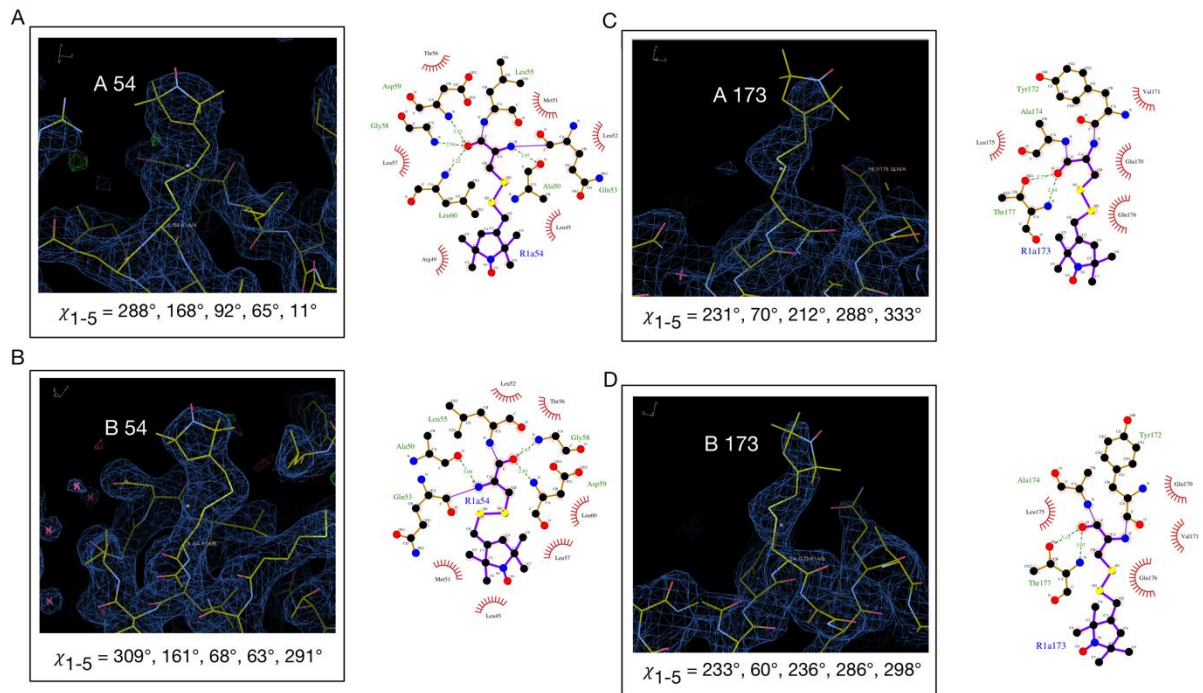
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3 **Supporting Figure 6: Superposition of spin labelled VcSiaP R125A Q54R1/L173R1 with**
4 **the VcSiaP wild-type.** Cartoon models of VcSiaP R125A Q54R1/L173R1 chain A (green)
5 and B (red), superimposed onto residues 1-100 of the VcSiaP wt structure (PDB-ID: 4MAG).
6 Chain A is almost identical to the wt structure, whereas chain B is in a slightly more closed
7 conformation.

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4 **Supporting Figure 7: Conformation of the R1 side chains in the VcSiaP R125A**
5 **Q54R1/L173R1 structure.** **A)** Left: Electron density (blue mesh, 2Fo-Fc contoured at 1.0 σ)
6 observed at the Q54R1 site (chain A). The protein is shown as yellow stick model. The dihe-
7 dral angles of the R1 side chain are given. Right: Ligplus scheme depicting the interactions of
8 the R1 side chain (purple) with its molecular environment. Covalent bonds are shown as solid
9 lines, polar interactions as dashed lines and nonpolar interactions as red arcs. Distances are
10 given in Å. **C-D)** same as A) but for the indicated R1 sidechains.
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4**Supporting Table 1**

[Neu5Ac _{tot}] (μ M)	VcSiaP open/close (%)		[closed] (μ M)			[open] (μ M)		
	Linear combination	Integration	Linear combination	Integration	Average	Linear combination	Integration	Average
0	100/0 (n.d.)	100/0	0.0 (n.d.)	0.0	0.0	25.0 (n.d.)	25.0	25.0
1	96/4 (\pm 0.9)	96/4	1.0 (\pm 0.2)	1.0	1.0	24.0 (\pm 0.2)	24.0	24.0
10	58/42 (\pm 0.6)	59/41	10.6 (\pm 0.2)	10.4	10.5	14.4 (\pm 0.2)	14.6	14.5
24	20/80 (\pm 1.8)	22/78	20.0 (\pm 0.5)	19.5	19.7	5.0 (\pm 0.5)	5.5	5.3
120	8/92 (\pm 1.2)	10/90	23.0 (\pm 0.3)	22.5	22.8	2.0 (\pm 0.3)	2.5	2.3
600	0/100 (n.d.)	3/97	25.0 (n.d.)	24.3	24.6	0.0 (n.d.)	0.7	0.4

#Values in parentheses represent the estimated error of the linear combination fitting procedure ($3*\sigma$).

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1 **Supporting Table 2**

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Mutant	Neu5Ac (mM)	VcSiaP Open/close (%)	
		Linear combination [#]	Integration
R125A	0	100/0 (± 1.2)	100/0
	1	100/0 (± 0.9)	99/1
	10	94/6 (± 1.8)	96/4
R125K	0	97/3 (± 0.9)	99/1
	1	85/15 (± 2.4)	88/12
	10	64/36 (± 3.0)	69/31
E184D	0	90/10 (± 2.1)	93/7
	1	8/92 (± 1.5)	9/91
E184Q	0	89/11 (± 3.3)	96/4
	1	37/63 (± 2.1)	39/61
E184A	0	100/0 (± 1.2)	98/2
	1	45/55 (± 0.9)	45/55
H207A	0	94/6 (± 3.0)	99/1
	1	4/96 (± 0.9)	7/93
H207Q	0	99/1 (± 3.0)	97/3
	1	5/95 (± 1.2)	7/93

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[#]Values in parentheses represent the estimated error of the linear combination fitting procedure (3σ)

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