Protein Motion and Configurations in a Form-Fitting Nanopore: Avidin in ClyA

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ABSTRACT We probe the molecular dynamics and states of an avidin protein as it is captured and trapped in a voltage-biased cytolysin A nanopore using time-resolved single-molecule electrical conductance signals. The data for very large numbers of single-molecule events are analyzed and presented by a new method that provides clear visual insight into the molecular scale processes. Avidin in cytolysin A has surprisingly rich conductance spectra that reveal transient and more permanently trapped protein configurations in the pore and how they evolve into one another. We identify a long-lasting, stable, and low-noise configuration of avidin in the nanopore into which avidin can be reliably trapped and released. This may prove useful for single-molecule studies of other proteins that can be biotinylated and then transported by avidin to the pore via their coupling to avidin with biotin-avidin linking. We demonstrate the sensitivity of this system with detection of biotin attached to avidin captured by the pore.

INTRODUCTION

It is possible to observe individual charged biological molecules as they translocate through a voltage-biased nanopore in a lipid membrane by monitoring the ionic conductance of the pore during the molecular motion through the pore. Such measurements have provided new insights into the biophysics of these molecules and their interactions with the pores (1). They have also resulted in practical, portable instruments for sequencing DNA (https://nanoporetech.com/). Probing proteins by this translocation method has provided interesting insights but proved challenging due to the more complex geometrical and charge structures of proteins; however, progress is being made (2–7).

We use an alternative method that involves studying proteins that do not pass through the pore but are rather transiently or more permanently trapped within a large pore by electrophoretic attraction. The method has been pursued recently (8,9) in cytolysin A (ClyA) nanopores and shown great promise. In the following, we show that when a very close match between the protein size and the pore lumen size is chosen, remarkably detailed, time-dependent electrical-conductance spectra are observed that reveal discrete protein configurations with the pore, including a low-noise and long-lasting trapped state that can be cleared deterministically with a control voltage.

The size-matched pair we have chosen is avidin in ClyA12, a dodecamer nanopore, because the molecular structures suggest the possibility of at least one highly constrained geometrical configuration (which we observe). Avidin is a positively charged protein with a high isoelectric point of roughly 10 (10,11), and its size is well understood from x-ray studies (12,13). The ClyA pore exists in several oligomeric forms, each having a different pore size and open-pore conductance, but the dodecamer pore is the most stable (14,15).

We also present, what is to our knowledge, a new method for analyzing and visualizing the data for very large numbers of single molecule events of protein trapping in a nanopore. The method provides clear views of the global dynamics and reveals molecular scale configurations and their evolution, even for relatively rare short events.

Finally, we demonstrate the sensitivity of the avidin ClyA12 system to molecular perturbations by detecting biotin linked to the avidin protein in the deeply trapped state.

MATERIALS AND METHODS

The protein-nanopore system

Fig. 1 A shows molecular models, with dimensions, of the avidin and dodecamer ClyA12 nanopore. The close match between the avidin outer dimensions and the pore lumen’s inner dimension makes this pair...
Avidin used in our experiments (Pierce Avidin; Thermo Fisher Scientific) has a polysaccharide attached to each of the four Asn17 locations. In addition to the core GlcNAc, the polysaccharides have four to five mannose and 2 GlcNAc (11).

**Experimental setup**

Fig. 1B illustrates the experimental setup. Two chambers of electrolyte solution (150 mM NaCl, 15 mM Tris at pH 7.5) are separated and electrically isolated from each other by a lipid bilayer of 1,2-diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL) stretched across a 40-micron diameter teflon frame. Ag/AgCl electrodes are inserted in each chamber and a voltage bias is applied across the membrane. For later trapping of positively charged avidin in the pore, the trans chamber is kept at positive voltage. ClyA nanopores are first added to the cis chamber. With a negative bias voltage applied, the current is transiently blocked when individual, positively charged avidin molecules are trapped by and subsequently escape or, by voltage reversal, are ejected from the pore.
ClyA monomer expression and purification

All reagents were purchased from Fisher Scientific and/or Boston Bioproducts (Ashland, MA) unless otherwise stated. Phenylmethane sulfonyl fluoride and magnesium chloride were purchased from Sigma (St. Louis, MO).

C-terminal His₆ tagged ClyAwt protein was expressed in BL21 (DE3) cells. Specifically, pT7-ClyAwt-CHis₆ plasmid was transformed in BL21 (DE3) chemically competent cells and grown on Luria-Bertani (LB)-Amp Agar plates. One colony was inoculated in starter LB media containing (DE3) chemically competent cells and grown on Luria-Bertani (LB)-Amp media containing 100 µg/mL ampicillin antibiotic and grown at 37°C with shaking at 200 revolutions per minute. The starter culture was used to inoculate 250 mL LB media containing 100 µg/mL ampicillin. The culture was grown at 37°C until the OD₆₀₀ was between 0.5 and 0.65. The culture was then cooled on ice and induced by adding isopropylthiogalactoside to a final concentration of 0.5 mM and the pelleted and resuspended in 15 mL of 50 mM Tris-HCl pH 8.0, 1 mM ethylenediaminetetraacetic-acid buffer and frozen in −20°C until ready to use.

The frozen pellet was subsequently thawed at room temperature and a final concentration of 0.5 mM phenylmethane sulfonyl fluoride was added. The mixture was sonicated on ice on lyse the cells. MgCl₂ was added to the lysate at a final concentration of 10 mM and the supernatant was centrifuged for 20 min at 20,000 × g. The supernatant was filtered through a 0.22 µm filter membrane and loaded onto a gravity nickel-nitrioltriacetic acid column equilibrated with buffer A (150 mM NaCl, 50 mM Tris-HCl pH 8). The column was subsequently washed with buffer A to remove unbound proteins. Buffer A1 (150 mM NaCl, 50 mM Tris-HCl, 50 mM imidazole) was used to wash the weakly bound proteins and then the ClyA protein was eluted and collected in buffer A2 (150 mM NaCl, 50 mM Tris-HCl, 50 mM imidazole).

The eluted ClyA proteins were dialyzed using a 6–8 kDa cutoff membrane with constant stirring at 4°C for two cycles in dialysis buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid). The proteins were then concentrated using a 10 kDa cutoff centricron to ~3 mL and loaded onto a gel filtration column equilibrated in 150 mM NaCl, 20 mM sodium phosphate pH 7.0 buffer to remove aggregated proteins. The ClyA monomer was collected and kept at 4°C for 2 weeks or in −80°C for long-term storage.

Preparation and purification of ClyA oligomers

Purified ClyA monomers were suspended at 0.6 mg/mL in a buffered solution containing 50 mM NaCl, 10 mM sodium phosphate pH 7.4 (with a buffer exchange column). Oligomeric ClyA was formed from monomers by the addition of n-Dodecyl β-D-maltoside (10% w/v in water; DDM, Calbiochem/EMD Millipore, Billerica, MA) to a final concentration of 1% and incubated 20 min at room temperature.

ClyA nanopore purification was carried out by blue native gel electrophoresis using a 4–16% polyacrylamide gradient gel (NativePAGE; Invitrogen/Novex Life Technologies, Carlsbad, CA). Typically, 10 µg of ClyA oligomers were combined with electrophoresis loading buffer and applied to a 1.0 × 5.0 mm sample well of the gel. Major bands of oligomeric ClyA were excised from the gel following electrophoresis, and nanopores were recovered from the gel slices by diffusion into an elution buffer containing 150 mM NaCl, 0.2% DDM, 50 mM Tris-HCl pH 8.0.

Preparation of avidin

Lyophilized purified avidin from hen egg white (Product# 21121; Pierce/Thermo Scientific) was dissolved in deionized water to 2 mg/mL concentration. For subsequent storage at 4°C, an equal volume of 2× Phosphate Buffered Saline with 20% glycerol was added to the suspension to bring the avidin stock solution concentration to a nominal 1 mg/mL. Before use in ClyA nanopore experiments, an aliquot of the avidin stock solution was applied to a Bio-Spin 30 spin column (Bio-Rad Laboratories, Hercules, CA) equilibrated with 150 mM NaCl, 15 mM Tris-HCl pH 7.5 for buffer exchange.

Preparation of d-biotin

Biotin was prepared with 0.2 mg d-Biotin (Sigma-Aldrich/Millipore Sigma) per mL of 20 mM KCl, 50 mM Tris-HCl pH 7.6, and diluted in 150 mM NaCl, 15 mM Tris-HCl pH 7.5 to a final biotin concentration of 100 µM.

RESULTS AND DISCUSSION

Avidin in a 1.66 nS pore

After 10 pmol of avidin is added to the 250 µL cis chamber with a 1.66 nS pore in place, the current through the –35 mV voltage-biased pore is observed to transiently drop from the open pore value (58 pA) as individual avidin protein molecules are captured by, and escape from, the pore (Fig. 2, A–G). These current blockage events are separated into two categories. The first we call transient captures. It consists of transient current blockage events that last from 200 µs up to 1 s before the pore current returns to the open-pore value after a captured protein has spontaneously escaped from the pore. The second category we call permanent captures. These events last for times exceeding 1 s, and the captured protein is almost never observed to spontaneously leave the pore. For these events, we automatically reverse voltage bias after 1 s of current blockage. This ejects the positively charged protein from the pore. The voltage bias is then returned to the original polarity (negative voltage on the trans electrode) and the open pore current is again observed, followed by new current blockade capture events. Thus, our permanent events all last for 1 s, at which time the trapped avidin is forcibly ejected from the pore.

Each single protein capture, whether transient or permanent, is observed to have its own unique time-dependent current trace during the event. Fig. 2, A–G show a collection of single molecule capture events in the 1.66 nS pore. We plot the observed current I(t) during each event, divided by the open pore current Iₒ. The events in Fig. 2, A–E show the time-dependent blockade for transient events of increasing duration. Most transient events are simple, having only a single blockade level as in the first event in Fig. 2 A. The longer transient events, Fig. 2, B–E, have been chosen to show that sometimes transitions between levels are observed during a single event. In a first-pass analysis, each transient capture is characterized by two numbers: the duration and the average blockade during the event. The “blockage” is defined to be (Iₒ–I(t))/Iₒ averaged over each event’s duration. Fig. 2 H is a “scatter plot” of these parameters for each transient capture event (green points) reflecting this characterization. (Each green point belongs to one protein capture). Scatter plots for other bias voltages are shown in Fig. S1.
FIGURE 2 Capture events for individual avidin molecules. (A–G) Time traces for capture events are shown. We show both data points that were filtered with a 10 kHz four pole Bessel filter (yellow) and data that have been post-processed with a 1 kHz low-pass eight-pole Bessel filter (red/maroon). The first five (A–E) are transient events of increasing duration. Some have a rich time structure, with discrete blockage levels and transitions between them. The last two time traces (F and G) are for permanent events. Here, an immediate current drop to an entrance blockage level is followed by a transition to a deep, permanent capture level with very low current fluctuations. For all permanent events, we apply a +100 mV voltage pulse to eject the avidin from the pore after 1 s of trapping. (H) Scatter plot of event blockage versus duration. Each transient event, shown as a green dot in the figure, is characterized by its duration and average blockage. Each permanent event, represented by a red dot, is characterized by the time spent at the entrance level, and the corresponding average blockage, before transition into the permanent, deep blockage level, AC80. The seven events in (A)–(G) are indicated in the figure. We also mark the blockage levels AC40, AC52, and AC57 discussed in the text and visible in one or more of the time traces in (A–G).
As the transient capture event duration increases from a few hundred microseconds to a few hundred milliseconds, the average blockage slowly increases. In the next figure we will show that the reason for this is the existence of multiple discrete blockage levels that are averaged over in each event in the scatter plot. A few examples of separate and clearly distinguishable blockage levels and transitions between them are already seen in Fig. 2. A–E, and these events are also numbered in the scatter plot.

**Fig. 2.** F and G show the current traces for two permanent captures. Each of these events has an interesting time structure. As with most permanent capture events, they start with an intermediate, fluctuating blockage level that is followed by a deep and quiet permanent blockage level. The difference in low-frequency current-noise characteristics between the intermediate level and the permanent blockage level it leads to is seen clearly in the 1 kHz post-filtered time traces (red/maroon data traces) in Fig. 2. F and G. Quantitatively, for the full-bandwidth (10 kHz) data, the power spectral density for the permanent level is nearly constant at frequencies below 3 kHz with a (square root) value of 10 fA/√Hz, whereas the power spectrum for the intermediate level exhibits a significant 1/f noise component. At 1 kHz, the current noise for the permanent trap level is 10 times smaller than the value for the intermediate level. It is also 20% smaller than that for the open pore, which in turn is within a factor of 2 of the Johnson noise limit.

Permanent captures are characterized by the time duration and average blockage during the intermediate part of the event. With these parameters, each permanent capture event is added to the scatter plot of Fig. 2 H (red). The average blockage is clearly independent of duration and close to that of the longest transient events. This immediately suggests that, before capture to a deep permanent trap state, the protein often passes through a particular intermediate state of variable duration. The permanent trap state may be of special importance for future protein studies as discussed below. The protein often stays trapped in this state even after the voltage bias has been removed but can always be cleared by a large reverse bias voltage.

**Protein dynamics landscape analysis**

A global view of these complex dynamical signals and the existence of discrete blockage levels and their time-dependent populations are revealed clearly and quantitatively in the protein dynamics landscape (PDL) analysis and plots that we introduce for the first time in connection with Fig. 3. To generate a particular histogram in Fig. 3 A, all transient events that last for times less than a specified \( \tau_{\text{trans}} \) are pooled. Then the amount of time spent (in units of 160 µs time bins) at each blockage level for all these events is determined and a histogram of the time spent at each level is generated. We refer to this histogram as a blockage spectrum. A collection of such blockage spectra, for a set of increasing \( \tau_{\text{trans}} \) values, is a PDL. Note that selecting data for small \( \tau_{\text{trans}} \) allows rare, short events to be visualized and quantified accurately.

The PDL here is obtained for a fixed bias voltage of –35 mV. The PDL plot reveals several peaks, corresponding to discrete blockage levels for avidin in ClyA. Note that deeper blockage levels last longer than shallower levels.
A similar analysis can be performed on permanent capture events, and the results are shown in Fig. 3B for the same bias voltage of −35 mV. Here, all permanent capture events are considered together and each histogram refers to the accumulated time (in units of 160 µs) a blockage value appears up until \( \tau_{\text{perm}} \) after a capture event starts (which is when avidin first enters the pore). The deepest-blockage level is maximally populated for the largest \( \tau_{\text{perm}} \).

A complete set of PDL plots is obtained from data like those of Fig. 3, A and B at different bias voltages and are shown in Fig. S2.

In the PDL plot of Fig. 3A, we see a single peak centered at 40% blockage for transient events lasting 2 ms or less. We call this level AC40 (AC for “avidin capture”). As longer events are included (i.e., as \( \tau_{\text{trans}} \) is increased), a second peak emerges. With careful fitting, we determine this level to be centered at 52% blockage and denote the level AC52. At 10 ms the two peaks have equal intensity, and at 30 ms, the AC52 peak dominates. For longer times, a separate (AC57) peak at 57% blockage dominates, and finally we observe a few very deep captures for transient events that last longer than 500 ms. Here the blockage level at 80% dominates (AC80). Gaussian fits to the spectra clearly reveal a fifth peak at 45% blockage (AC45), and the fits are shown in Fig. S3.

It is interesting to compare this dynamical behavior to that revealed by the PDL plot for permanent events at −35 mV (Fig. 3B). During the first 2 ms of the permanent events, the spectrum is dominated by one peak: AC57. The AC40 peak is barely visible, and AC52 is missing. The spectra are generally dominated by AC57 during the early times of the permanent events, but later (≥100 ms) the AC80 peak dominates. So for the permanently trapped events, avidin is first captured into state AC57, where it stays for an average of 60 ms (at −35 mV). This is followed by capture into a deeply and permanently trapped state, AC80. This picture is supported by the time traces for the Permanent Capture events in Fig. 2, F and G.

This difference between the peak-structure dynamics in the PDL plots for transient and permanent events—in particular the absence of the low-blockage peaks AC40, AC45, and AC52 in the PDL plot for permanent events—indicates that for avidin to reach the permanent trap state AC80, it has to enter the pore in a particular orientation. To illustrate, we show in Fig. 4 A a 3D printed macroscopic model of avidin (black) in a ClyA12 pore (red). The pore is partially cut out to aid in visualization. (A) Orientation of avidin that does not allow for entry into the pore is shown. (B) An orientation of avidin that allows it to be deeply inserted in the form-fitting pore leading to the deeply trapped state.

Finally and importantly, we have demonstrated the sensitivity of the avidin-ClyA platform for detecting other molecules attached to avidin. Here 100 pmol of biotin were positively charged avidin further into the pore and result in the stably trapped state with 80% blockage (AC80).

We also observe that the level populations in the PDLs are strongly voltage-bias-dependent (Fig. S2). This effect is already dramatically seen by simply plotting the ratio of the number of permanent to transient events as a function of bias voltage. This is shown in Fig. 5. Increasing the bias voltage from 30 to 50 mV results in dramatic and exponential increase in this ratio. At high bias voltage, the energy landscape of the protein in the pore is clearly biased toward permanently capturing the avidin to the deepest level in the pore from which escape eventually becomes impossible.

FIGURE 4 Avidin trapped in ClyA12. We show a three-dimensional printed macroscopic model of avidin (black) in a ClyA12 pore (red). The pore is partially cut out to aid in visualization. (A) Orientation of avidin that does not allow for entry into the pore is shown. (B) An orientation of avidin that allows it to be deeply inserted in the form-fitting pore leading to the deeply trapped state.

**Biotin-avidin complex in a 1.66 nS pore**

Finally and importantly, we have demonstrated the sensitivity of the avidin-ClyA platform for detecting other molecules attached to avidin. Here 100 pmol of biotin were
added to the cis chamber already containing 10 pmol of avidin at –35 mV voltage bias. After a few minutes, we observed a dramatic change in the trapping dynamics. The ratio of permanent to transient event rates was reduced by a factor of 28, from 1/6.4 (avidin) to 1/182 (biotin-avidin), with the latter closely resembling the ratio for avidin alone at −30 mV bias voltage (1/159). From this we conclude that the net positive charge of the biotin-avidin complex is lower by a factor of 30/35 compared to avidin alone. Calculations (19) based on the protein data bank crystallographic structures for deglycosylated avidin (1AVE) and for the complex of deglycosylated avidin with four biotin ligands (2AVI) predict a charge of the biotin-avidin complex that is lower by two positive charge units than that of avidin alone. (Note, in both these cases avidin is deglycosylated except for the core glucosamine (GlcNAc)). Combining this with our observations, we obtain a charge of +14e for fully glycosylated avidin (used in our experiments).

Interestingly, we also observe that for the biotin-avidin complex, the blockage level for the deeply trapped state is decreased by 4% relative to that for avidin alone. This is shown in Fig. 6. Since the deep blockage level for avidin is independent of voltage in the −30 to −35 mV range, the difference in deep blockage levels could be due to a slightly bigger size or lower elasticity of the biotin-avidin complex (12,21). An alternative explanation is a weaker electrostatic attraction of biotin-avidin to the negative charges at the limiting aperture of ClyA.

CONCLUSIONS

Our experiments and analysis show that the avidin-ClyA nanopore system can be electronically probed and quantified in great detail. Discrete conductance levels, transitions between them, and the fluctuations within them all reflect the interaction between, and time evolution of, avidin and the voltage-biased pore in an electrolyte environment. One may expect that the discussions we have presented will be made more precise when the powerful computational tools of molecular modeling and simulation of ionic conduction in confined spaces are applied to this system (22). An important motivation for focusing on the avidin-ClyA system is the potential it presents for using avidin as a shuttle to bring other, biotinylated biological molecules linked to avidin to the pore for probing. Here, the deeply trapped state will be of special importance, allowing for probing at the single-molecule level over controllable time intervals against a low-noise background. A first step in this direction has already been taken by our detection of biotin attached to deeply captured avidin.

SUPPORTING MATERIAL

Supporting Materials and Methods, three figures, and three tables are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(18)30910-X.

AUTHOR CONTRIBUTIONS

B.L. contributed experimental work and computing assistance at Harvard. C.S. prepared three-dimensional protein models and performed experiments at Harvard. M.F. prepared ClyA at Amherst. M.C. directed work on expression and purification of ClyA at Amherst. J.A.G. and L.V.H. conceived the idea for the experiment and method of analysis, and directed research at Harvard, and in addition L.V.H. conducted experiments at Harvard.

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REFERENCES

Supplemental Information

Protein Motion and Configurations in a Form-Fitting Nanopore: Avidin in ClyA

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Supporting Material

Protein Motion and Configurations in a Form-Fitting Nanopore: Avidin in ClyA


Scatter Plots for Different Bias Voltages.

The scatter plot for a bias voltage of -35 mV is shown in the main text (Fig. 2h). In Figure S1 we show scatter plots for bias voltages of -50 mV, -40 mV, and -30 mV.

Figure S1: Scatter plots for Transient and Permanent Capture Events for bias voltages of -50 mV, -40 mV and -30 mV.
PDL Plots for Different Bias Voltages.

The PDL plots for Transient and Permanent Captures are shown in the main text for -35 mV. In Figure S2 we show PDL plots for bias voltages of -40 mV (A) and -30 mV (B). Fitting parameters for the plots are indicated in tables below the figures.

Figure S2 (A): PDL plots for transient and permanent captures for voltage -40 mV. Table S1 (below) shows the number of transient events for each blockage spectrum in the PDL plot for transient events. Also listed is the number of permanent events in the PDL plot for these events.

Table S1

<table>
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<th>$\tau_{\text{trans}}$ (ms)</th>
<th>2ms</th>
<th>5ms</th>
<th>10ms</th>
<th>30ms</th>
<th>100ms</th>
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<td>Number of transient events</td>
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<td>35</td>
<td>48</td>
<td>73</td>
<td>88</td>
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Total number of permanent events at -40mV is 139.
Figure S2 (B): PDL plots for transient and permanent captures for voltage -30 mV. Table S2 (below) shows the number of transient events for each blockage spectrum in the PDL plot for transient events. Also listed is the number of permanent events in the PDL plot for these events.

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<th>$\tau_{\text{trans}}$</th>
<th>2ms</th>
<th>5ms</th>
<th>10ms</th>
<th>30ms</th>
<th>100ms</th>
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Total number of permanent events at -30mV is 131.
Determination of Blockage Levels AC40, AC45, AC52, and AC57.

The positions of discrete blockage levels are obtained from fits to blockage spectra in a PDL plot. For the PDL plot for transient events at -35 mV (Fig. 3a in the main text), we obtain the levels below the 80% permanent level (AC80) by fitting a sum of four Gaussians to each spectrum for blockages below 65%.

For efficient convergence of the fitting routine, we use the following procedure:

First the spectrum for $\tau_{\text{trans}} = 1000\text{ms}$ is considered. We fit one Gaussian to the spectrum in a restricted blockage region around the dominant 57% peak, and then subtract the resulting Gaussian from the data. The result of this subtraction shows the 52% peak clearly and we fit with a single Gaussian in a blockage region around that peak and again subtract the resulting Gaussian. This reveals the 45% peak which is fitted with a Gaussian in a restricted blockage region. The resulting values for the position and width of the two Gaussians centered at 57% (AC57) and 45% (AC45) are then used as fixed values for fits with four Gaussians to all spectra over the whole blockage region below 65%. The result of the fits and the values of positions, widths, and amplitudes of each Gaussian component are shown in Fig. S3.

**Figure S3:** (see below) Gaussian fits to the PDL blockage spectra at -35 mV. The data (red dots), fit function (solid blue curve), and the four individual Gaussians are shown for each blockage spectrum, with $\tau_{\text{trans}}$ indicated in Figures S3, A-G.
The results of the fitting procedure for the position (Pos), width (Sigma), and amplitude (Amp) of each peak and for each spectrum are shown in Table S3, below. The table is below all the plots.
Figure S3
Figure S3
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<tr>
<th>$\tau_{\text{trans}}$</th>
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<th>AC45</th>
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<td></td>
<td>Amp</td>
<td>Pos</td>
<td>Sigma</td>
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<td>1000</td>
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AC45 Pos = 0.4501 ±0.002, AC45 Sigma=0.0252 ±0.002
AC57 Pos = 0.5679 ±0.001, AC57 Sigma=0.0296 ±0.001