Karen Glass AUC Analysis

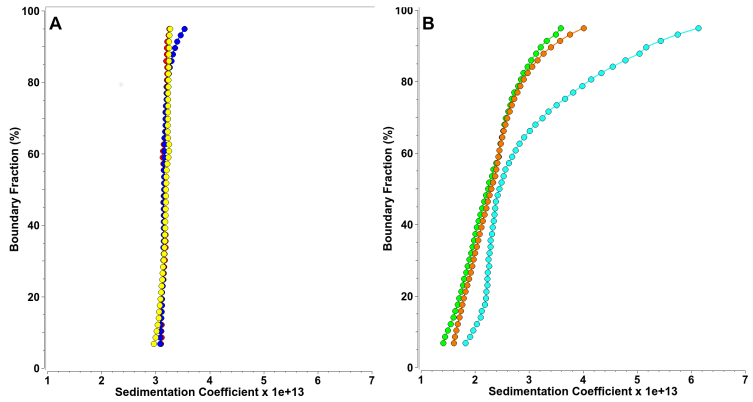

*Figure 1: van Holde-Weischet plot showing sedimentation distributions of PfBDP1 (**A**) and CECR2 (**B**). For PfBDP1, low (red), medium (yellow), and high (blue) concentrations have homogeneous profiles that overlap. CECR2 low (green), medium (orange), and high (cyan) concentrations are quite heterogeneous. In the high CECR2 concentration, some self-association is present.*

Structure and Function of the PfBDP1 and CECR2

Samples of PfBDP1 and CECR2 were prepared for analytical ultracentrifugation (AUC) for the purpose of checking for self-association. From the stock solutions three concentration were made, determined by a ThermoFisher Genesys 10s benchtop spectrophotometer and the provided extinction coefficients at 280 nm. For PfBDP1, 3.13 μM, 10.46 μM, and 21.37 μM concentration were made, and for CECR2, 10.46 μM, 26.16 μM, and 68.52 μM. Low and medium concentrations were loaded into standard 1.2 cm Epon centerpieces, and due to stock material being limited, the high concentrations were loaded into a 3 mm Titanium centerpiece. The experiment was performed at 35,000 rpm for 12 hours at 5°C

Sedimentation profiles for all concentrations are shown in Figure 1. PfBDP1 concentrations are homogeneous, based on the near vertical integral sedimentation coefficient profiles. Furthermore, all PfBDP1 concentrations produced overlapping distributions at a single sedimentation value, 3.08 S, indicating good homogeneity and absence of mass action. WIn contrast, all CECR2 concentrations were quite heterogeneous. In the high CECR2 concentration, a shift in sedimentation profile suggests self-association, further highlighted in a pseudo-3D plot (Figure 2). Around 68% of the total signal is from the monomeric species and 27% from the larger oligomer, suggesting that the majority of the highest concentration still remains monomeric. It is noteworthy that the frictional ration of the oligomeric form is close to one, suggesting that the oligomeric form is rather globular. Hydrodynamic parameters for the observed monomeric and oligomeric CECR2 and the monomeric PfBDP1 are listed in Table 1.

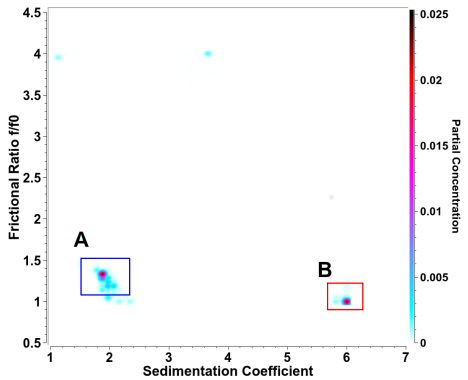

*Figure 2: Pseudo-3D plots of frictional ratio as a function of sedimentation coefficient of high CECR2 concentration. This plot was generated using a 2D Spectral Analysis Monte Carlo 50 iteration model. This plots shows the monomeric species (**A**) and a larger oligomer (**B**).*

Table 1: Characterization of PfBDP1 and CECR2 proteins

	PfBDP1	*CECR2 (A)*	*CECR2 (B)*
S (x 10^{-13} s)	3.08	1.94	5.99
D (x 10^{-6} cm^2/s)	4.83	1.02	7.93
M (kDa)	56.41	16.83	66.75
f/f_0	1.75	1.25	1.01

** **A** and **B** corresponds to the boxes in Figure 2*

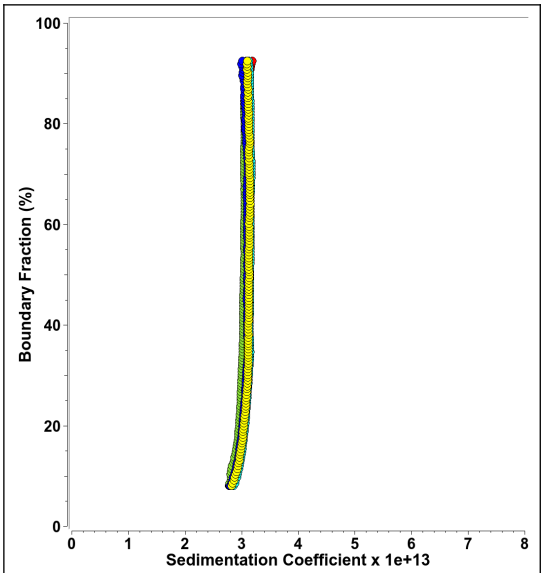

Figure 3: van Holde-Weischet plot of PfBDP1 and peptide ligands showing the distributions of sedimentation coefficients. PfBDP1 control (red), PfBDP1+H4K16ac 1:5 (green), PfBDP+H4K 1:1 (blue), PfBDP1+H2B 2:1 (cyan), PfBDP1+H2B 1:2 (yellow).

Interaction of Full Length PfBDP1 with Acetylated Histone Ligands

The goal of this project was to determine, using AUC, whether histone ligands interact with full length PfBDP1, and if this interaction results in oligomerization. Two different multi-acetylated histone H4 ligands, H4K5acK8acK12acK16ac (H4K16ac) and H2B.ZK3acK8acK13acK14acK18ac (H2B), at two different molar ratios with PfBDP1 were tested (PfBDP1+H4K16ac 1:5, PfBDP+H4K 1:1, PfBDP1+H2B 2:1, PfBDP1+H2B 1:2). The amount of material sent for this experiment was insufficient to test for mass action at higher protein concentrations in the presence of peptides.

150 µL of pre-assembled samples were received along with buffer, consisting of 20 mM NaPO4 (pH7.5), 150 mM NaCl, and 1.0 mM TCEP. The buffer was used to dilute the samples to 450 µL to provide sufficient volume for the AUC experiment. Using a ThermoFisher Genesys 10s benchtop spectrophotometer, absorbance spectra for each sample were collected from 210-315 nm. There was some absorbance seen above 300 nm (0.2 OD at 315 nm), suggesting that some of the PfBDP1 was aggregated. Because centrifugation will pellet the aggregated protein rapidly, potentially leaving a low protein concentration. For this reason, 237 nm and 280 nm were measured to ensure there would be enough absorbance once the aggregates were sedimented. Due to the presence of TCEP, measurements below 237 nm incur too much background absorbance, limiting the wavelength range of observation. The run was spun at 35,000 rpm at 5°C for 12 hours.

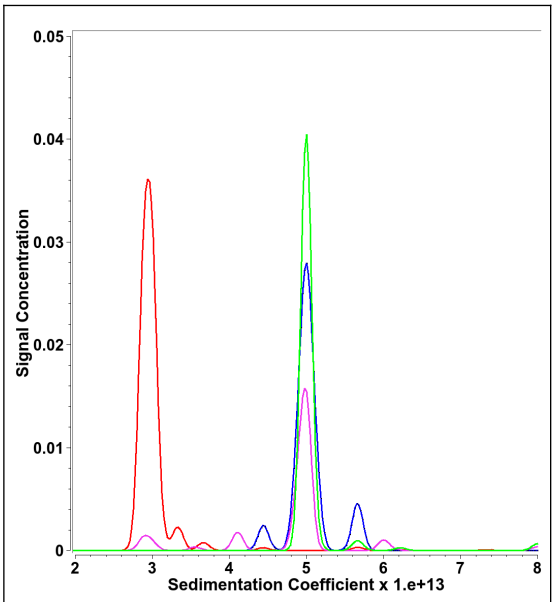

Figure 4: Sedimentation distribution of PfBDP and DNA using 2DSA-IT models. The pure protein (red) and the pure DNA (blue) are compared to the PfBDP1-DNA 1:3 complex at two different wavelengths, 230 nm (magenta) and 260 nm (green).

Figure 3 shows the sedimentation profiles of PfBDP1 and its mixtures with H2B and H4K16ac ligands. All mixtures examined display the same sedimentation profile, showing no significant change in sedimentation pattern with the presence of either peptide. This suggests that at this protein concentration the presence of ligands does not influence the oligomerization of PfBDP1, and that they are unlikely to bind to PfBDP1 at these concentrations.

Interaction of Full Length PfBDP1 with DNA Ligand

A PfBDP1-DNA mixture was also measured to determine the presence of ligand mediated oligomerization. This experiment was designed to compare the behaviour of PfBDP1 in the presence and absence of DNA. The DNA binding was tested using a 1:1 and 1:3 ratio of protein:DNA. Unfortunately, the volume and concentration sent were too low to design a successful experiment, and limited our ability to determine a PfBDP1 extinction spectrum, which is needed for the multi-wavelength AUC deconvolution. The protein, the DNA, and their mixture were measured at 280 nm for the protein control, at 260 nm for the DNA control, and their mixture was measured

as a MW-AUC experiment from 228-280 nm in 2 nm increments. Because of the lack of a suitable protein extinction spectrum (due to lack of sample), these data however could not be analyzed as a multi-wavelength experiment.

Figure 4 compares the sedimentation distributions of PfBDP1, DNA, and the PfBDP1-DNA 1:3 mixture. Unfortunately, a multi-wavelength (MW) deconvolution of the MW-AUC data was not possible because the signal from the protein was too low. Examining the 230 nm and 260 nm wavelengths from the mixture, we see that at 230 nm there is a small peak that aligns with the protein control and a much larger peak that aligns with the DNA control. At 260 nm, only a single peak that aligns with the control DNA is seen. We also do not see any peak larger than the DNA control peak, which would be expected if binding were occurring at this concentration. This pattern is further seen in Figure 5, a 3D sedimentation plot using the multi-wavelength data from the two PfBDP2-DNA ratios. All significant signal is concentrated around 5 S, again aligning with the DNA control, very similar results were also obtained for the 1:1 mixture. Because of the negligible signal of the protein, and the mixmatch of the concentrations between protein and DNA (a 1:3 DNA:Protein mixture would have been more informative than a 3:1 mixture), it cannot be confirmed that the protein is binding to the DNA. The absence of a presumably faster peak for a potential complex does not mean that there is no interaction, it just means that it cannot be seen with the current protein:DNA ratios. We could try again with a shorter DNA (to reduce DNA extinction) and/or higher protein concentration.

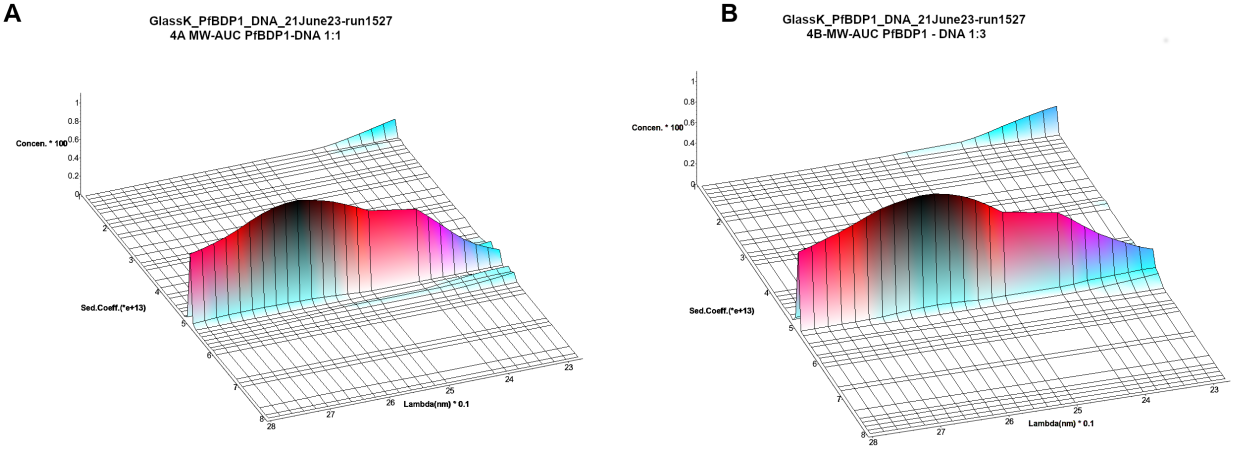

Figure 5: 3D multi-wavelength plot of PfBDP1- DNA 1:1 (A) and PfBDP1-DNA 1:3 (B) displaying the frequency of sedimentation coefficients from 230-280nm. From both samples only one significant peak is seen aligning with the DNA control.

Interaction of CECR2 BRD with Acetylated Histone Ligands

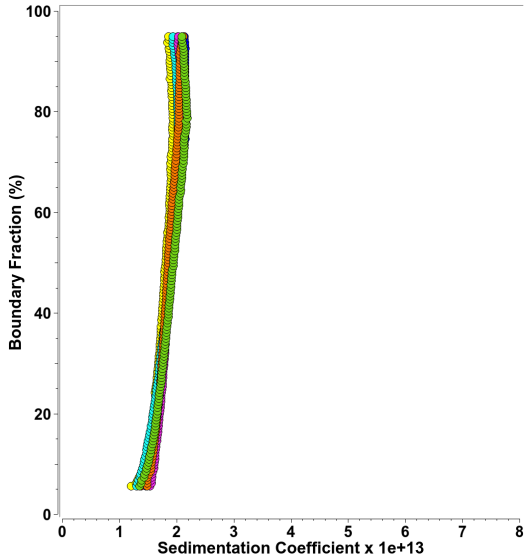

Figure 6: van Holde-Weischet plot of CECR2 with peptide ligands. CECR2 control (red), CECR2+H3K18ac 1:2 (blue), CECR2+H3K18ac 1:1 (yellow), CECR2+H3K18ac 2:1 (magenta), CECR2+H3K14ac 2:1 (orange), CECR2+H4K16ac 1:1 (cyan), and CECR2+H4K8ac 2:1 (green).

Similar to the run with PfBDP1 and subsequent acetylated histone ligands, we carried out an AUC experiment testing for oligomerization when stoichiometric mixtures of CECR2 BRD protein with H4K8ac, H4K5acK8acK12acK16ac (H4K16ac), H3K14ac, and H3K4acK9acK14acK18ac (H3K18ac) ligands are measured. Each sample was received as a 150 µL pre-assembled solution and brought up to a volume of 450 µL using the provided buffer for AUC measurements. The absorbance spectra showed no absorption above 300 nm, inferring that there is no aggregation of the protein. The protocol design was identical to the PfBDP1 experiment, run at 35,000 rpm for 12 hours at 5°C.

All CECR2 peptide sedimentation distributions are displayed by overlaying van Holde-Weischet sedimentation coefficient distributions (Figure 6), and similar to the PfBDP1 peptide samples, all profiles overlap. All peptide mixtures with CECR2 sediment identical to the CECR2 control, indicating that CECR2 remains monomeric in the presence of histone peptides. Because the peptides lack a unique chromophore, we cannot resolve the question whether the peptides actually bind to the protein.