Supporting information for

Avidity for polypeptide binding by nucleotide-bound Hsp104 structures.

Experimental Procedures

Reagents and Buffers

All chemicals were reagent grade. All buffers were prepared with distilled, deionized water from a Purelab Ultra Genetic system (Evoqua, Warrendale, PA). Buffer HK150 contains 25 mM HEPES, pH 7.5 at 25 °C, 150 mM KCl, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 10% (v/v) glycerol. ATP and ADP were purchased from Thermo Fisher Scientific (Waltham, MA). AMPPCP was purchased from Sigma-Aldrich (Darmstadt, Germany). ATPγS and AMPPNP were purchased from CalBiochem (La Jolla, CA).

Protein and Peptide

Hsp104 and Hsp104A503S were purified as described.[1,2] Protein concentrations are reported in monomer units. Hsp104 concentration was determined spectroscopically using ε280 = 32,500 M^{-1} cm^{-1}.

ClpB was expressed with an N-terminal His_6tag, followed by a TEV cleavage site for purification purposes. The mutagenesis was performed by GenScript (Piscataway, NJ). The PET-30a(+) plasmid was transformed into OneShot ® *E. coli* BL21(DE3) (Invitrogen) cells following the manufacturer's protocol. Protein was expressed in *E. coli* BL21(DE3) cells under *lac* operon control and with kanamycin resistance. Cell paste was suspended in cell lysis buffer [40 mM Tris, pH 7.5, at 4°C, 500 mM NaCl, 10% (w/v) sucrose, 20% (v/v) glycerol, 20 mM imidazole, 2 mM 2-mercaptoethanol]. Cells were lysed by two passes through a French press. DNase and RNase were added to the lysate and incubated with stirring at 4 °C for 15 minutes. Cell debris was pelleted by subjecting the lysate to centrifugation at 28,000 g in a ThermoScientific Fiberlite F14-6x250 rotor at 4 °C for 120 min. His_6ClpB was isolated by batch purification using GE Healthcare Bio-Sciences AB (Uppsala, Sweden) Ni SepharoseTM 6 Fast Flow charged media. The loose beads were incubated with the supernatant for 75 minutes at 4 °C with stirring and subsequently washed with 20-30 column volumes (where one column volume is defined as the volume of loose media used) nickel buffer 1 (40 mM Tris, pH 7.5 at 4 °C, 500 mM NaCl, 10% (v/v) glycerol, 20 mM imidazole, 2 mM 2-ME). The media, with bound protein, was then incubated with 1.5 CV of nickel buffer 2 (40 mM Tris, pH 7.5 at 4 C, 500 mM NaCl, 10% (v/v) glycerol, 500 mM imidazole, 2 mM 2-ME) for 30 minutes at 4 °C with rocking. The slurry was poured into a gravity column. The eluent was collected and the media was washed with an additional 6-7 column volumes of nickel buffer 2. His_6ClpB was dialyzed into digestion buffer (25 mM Tris pH 7.5 at 4 °C, 150 mM NaCl, 20% (v/v) glycerol, 500 uM EDTA, 2 mM 2-ME) in 15 KDa

cut off tubing. The His_6-tag on ClpB was cleaved by His_6 tagged Tobacco Etch Virus (TEV) protease (preparation described below) in a ratio of 27 mg His_6ClpB : 1 mg His_6-TEV. After digestion, the sample was dialyzed into nickel buffer 3 (40 mM Tris, pH 7.5 at 4 °C, 500 mM NaCl, 10% (v/v) glycerol, 500 mM imidazole, 2 mM 2-ME) and run over a GE Healthcare (Little Chalfont, UK) HisPrep FF 16/10 column. After washing with nickel buffer 3, ClpB from which the His_6 tag had been cleaved, was eluted with nickel buffer 1 (20 mM imidazole). His_6TEV, undigested His_6ClpB, and any remaining His_6 tags cleaved from the protein were eluted with nickel buffer 2 (500 mM imidazole). Note that the digested ClpB includes an N-terminal glycine residue that remains from the TEV cleavage site. The purity of ClpB was assessed to be greater than 95% by SDS/PAGE with Coomassie Brilliant Blue staining. Sequence was confirmed by mass spectrometry. ClpB was dialyzed into storage buffer (25 mM Tris pH 7.5 at 4 °C, 500 mM NaCl, 50% (v/v) glycerol, 500 uM EDTA, 2 mM 2-ME) in 50 kDa cut off tubing and stored at -80 °C until use. Protein concentrations are reported in monomer units using the extinction coefficient at 280 nm ε = 35,562 M^{-1} cm^{-1} determined in our lab by the method proposed by Edolhoch and later refined by Pace *et al.*[3,4]

TEV protease with an N-terminal His_6tag was expressed from glycerol stocks kindly provided by Dr Bingdong Sha, UAB. The *E. coli* cells were resistant to ampicillin and chloramphenicol. A starter culture grown at 37 °C was used to inoculate a 1 L growth that continued growing at 37 °C, 225 rpm until OD600 reached ~0.6. Expression was induced by adding 1 mM IPTG and the temperature was lowered to 20 °C while shaking (225 rpm) continued overnight. Cells were harvested by centrifugation (6.5 g cell paste from 1 L growth). Cell paste was suspended in cell lysis buffer [40 mM Tris, pH 7.5, at 4°C, 500 mM NaCl, 10% (w/v) sucrose, 5% (v/v) glycerol, 2 mM 2-mercaptoethanol]. Cells were lysed by sonication (12 cycles of 15 second pulse, 45 second rest). Cell debris was pelleted by subjecting the lysate to centrifugation at 28,000 g in a ThermoScientific Fiberlite F14-6x250 rotor at 4 °C for 90 min. His_6TEV protease was isolated by batch purification using GE Healthcare Bio-Sciences AB (Uppsala, Sweden) Ni SepharoseTM 6 Fast Flow charged media. The loose beads were incubated with the supernatant for 120 minutes at 4 °C with stirring and subsequently washed with 20-30 column volumes (where one column volume is defined as the volume of loose media used) nickel buffer 1. The media, with bound protein, was then incubated with 1.5 CV of nickel buffer 2 for 30 minutes at 4 °C with rocking. The slurry was poured into a gravity column. The eluent was collected and the media was washed with an additional 10 column volumes of elution buffer though the protein eluted within the first ~4 column volumes. The purity of His_6TEV protease was assessed to be greater than 95% by SDS/PAGE with Coomassie Brilliant Blue staining and the protein was determined to be ~ 1 mg/mL (40 mg total yield) based on absorbance at 280 nm and an extinction coefficient of ε = 32,290 M^{-1} cm^{-1}. His_6TEV protease was dialyzed into storage buffer in 15 kDa cut off tubing and stored at -80 °C until use, which is recommended to be within one year of purification (conversation with Dr Jingzhi Li, UAB).

The peptide, NCysRepA50mer (C MNQSFISDIL YADIESKAKE LTVNSNNTVQ PVALMRLGVF VPKPSKSKGE), was synthesized by CPC scientific (Sunnyvale, CA). Note that the cysteine residue was added for labeling purposes and is not part of the native RepA sequence. The subsequent 50 amino acids are the first 50 amino acids of the RepA sequence. The peptide was fluorescently modified

using fluorescein-5-maleimide, purchased from Life Technologies (Carlsbad, CA). The unmodified peptide was dissolved in 6 M guanidine hydrochloride, 20 mM HEPES, pH 7 at 25 °C and dialyzed against the same buffer. Fluorescein-5-maleimide was dissolved in 6 M guanidine hydrochloride, 20 mM HEPES, pH 7 at 25 °C to prepare a 30 mM solution. Tris(2-carboxyethyl)phosphine (TCEP) was dissolved in 6 M guanidine hydrochloride, 20 mM HEPES, pH 7 at 25 °C. These reagents were combined such that a 500 μL reaction mixture contained 100 μM peptide, 2 mM dye, and 1 mM TCEP. The labeling reaction proceeded for three hours at ambient temperature under partial vacuum. The labeled peptide was isolated using a GE Healthcare (Little Chalfont, UK) Superdex Peptide 10/300 GL column with absorbance monitored at 230 nm, 280 nm, and 495 nm. The column was run in an isocratic gradient of 6 M guanidine hydrochloride, 20 mM HEPES, pH 7 at 25 °C. The peptide was dialyzed into buffer HK150.The modified peptide is referred to as FluNCysRepA50mer.

α-Casein that was used as a protein trap in stopped flow experiments was purchased from Sigma-Aldrich (Darmstadt, Germany). The protein was dissolved in 6 M guanidine hydrochloride, 20 mM HEPES, pH 7 at 25 °C and dialyzed into HK150 using 1 kDa cut off tubing and stored at -20 °C until use. Protein concentrations were determined using the extinction coefficient at 280 nm $\varepsilon = 24{,}500\ M^{-1}\ cm^{-1}$.

Sedimentation Velocity Experiments

Sedimentation velocity experiments were performed on Hsp104 in a Beckman ProteomeLab XL-I analytical ultracentrifuge using the interference optical system (Beckman Coulter, Brea, CA). Experiments were carried out by loading 390 μL of sample and reference solutions into each corresponding sector of a 12 mm double sector Epon charcoal-filled centerpiece. Samples were subjected to an angular velocity of 40,000 rpm and scans were collected every 30 s at 25 °C.

Sample and reference solutions for each sedimentation velocity experiment were supplemented with identical concentrations of nucleotide to eliminate contributions from nucleotide to the signal. Protein samples were prepared by incubating 2 μM Hsp104 with either no nucleotide, or a fixed concentration of 300 μM or 2 mM of each of the four nucleotides tested: ATPγS, AMPPNP, ADP, and AMPPCP. Protein and reference solutions were prepared in HK150 and incubated for 2 hours at 25 °C before the first sedimentation scan was collected. Each experiment was performed with protein no older than two days from the time of dialysis.

Analysis of Sedimentation Velocity Experiments

Interference boundaries acquired from sedimentation velocity experiments were first corrected for time stamp errors[5,6] using REDATE Version 0.1.7 (Chad Brautigam, University of Texas Southwestern Medical Center). The corrected boundaries were then analyzed using SEDFIT version 14.4f (Peter Schuck, NIH) as previously described.[7,8] The data were analyzed between the meniscus plus 0.01 cm and 6.7 cm to minimize the contribution from gradients of glycerol and nucleotide to the sedimentation boundaries.[7] The weight

average sedimentation coefficients reported in Table 1 were obtained by integrating the c(s) distributions over the range of $s_{20,w}$ ~14 – 20 S. For each nucleotide, the sedimentation coefficients obtained from the two nucleotide concentrations tested in duplicate were averaged. The corresponding standard deviations are reported in Table 1. All sedimentation coefficients, s, are reported as $s_{20,w}$ by correcting s to the standard solution condition of water at 20 °C, as previously described, using Eq. (S1):[7,9]

$$s_{20,w} = \frac{\left(1-\rho_{20,w}\bar{v}\right)}{\left(1-\rho\bar{v}\right)} \cdot \frac{\eta}{\eta_{20,w}} \cdot s \qquad \text{(S1)}$$

where ρ is the density of the buffer, $\bar{v}$ is partial specific volume of the protein, and η is viscosity. The partial specific volume for Hsp104 was calculated to be 0.7398 mL g^{-1} from the protein sequence using Sednterp[10] (David Hayes, Magdalen College, Tom Laue, University of New Hampshire, and John Philo, Alliance Protein Laboratories). A correction of $\Delta\bar{v}$ = +0.0033 mL g^{-1} (Eq. (S2))[11] was added to obtain a final value of $\bar{v}$ = 0.7431 mL g^{-1}, that accounts for changes in hydration due to the presence of 10 % glycerol in the buffer used here, as previously done by us to examine the hydrodynamics of ClpA[12] and ClpB[7].

$$\frac{\Delta\bar{v}}{\Delta[\text{glycerol}]\%(\text{v/v})} = \left(3.33 \pm 0.38\right) \times 10^{-4} \text{ mL g}^{-1} \qquad \text{(S2)}$$

Analysis of Frictional Ratios

The ratio of the frictional coefficient (*f*) of Hsp104 relative to the frictional coefficient of a hydrated sphere of equal mass (f_0), can be used to gain insight into how the sedimentation coefficient correlates to structure. Frictional coefficient ratios (f/f_0) were determined using weight average sedimentation coefficients for Hsp104 (Table 1). The frictional ratios were calculated using the measured $s_{20,w}$ as done previously by us to investigate the self-assembly of ClpA[13] using Eq. (S3):

$$\frac{f}{f_0} = \left(\frac{M^2\left(1-\bar{v}\rho\right)^3}{162\pi^2 S_{20,w}^3 \eta^3 N_A^2 (\bar{v} + \delta\bar{v}_{H_2O})} \right)^{\frac{1}{3}} \qquad \text{(S3)}$$

where *f* is the frictional coefficient for Hsp104, f_0 is the frictional coefficient of a hydrated sphere of equivalent mass, *M* is the molecular weight of Hsp104 (102,648 g/mol), N_A is Avogadro's number, and δ is the degree of hydration of Hsp104 (grams of water bound per gram of Hsp104). The degree of hydration was determined from sequence using Sednterp to be 0.4342 g. As done previously by us, a correction

was applied to the degree of hydration value to account for the observation that only 70 % of the degree of hydration calculated using the Kuntz method is associated with a folded protein.[13,14] After this correction is applied, the degree of hydration of Hsp104 is approximated to be 0.3039 g of water bound per macromolecule. The resulting values for the frictional ratio obtained for Hsp104 in the presence of each nucleotide condition tested are summarized in Table 1.

Fluorescence Anisotropy

Steady state fluorescence anisotropy was monitored by exciting fluorescein at 494 nm and observing emission at 515 nm in a Fluorolog-3 spectrophotometer (HORIBA Jobin Yovin, Edison, NJ) as previously described.[15] Measurements were made with FluNCysRepA50mer alone, then upon subsequent additions of various nucleotides, and finally addition of Hsp104. Final concentrations were 20 nM FluNCysRepA50mer, either 300 μM or 2 mM nucleotide, and 2 μM Hsp104. Note that anisotropy is not concentration dependent so the minor changes in concentration due to sequential additions of components does not contribute to the anisotropy signal. Experiments were performed at 25 °C in buffer HK150.

For each data set presented, the average and standard deviations are reported as follows. The anisotropy value for peptide alone is the average of all time points from all samples which contain only peptide. For example, the anisotropy value $r = 0.060 \pm 0.004$ reported in the main text for free peptide in buffer is the average and standard deviation of 50 total time points, ten time points from the "no nucleotide" sample, Figure 2 black triangles (from time 0 to 80 minutes) and five time points from each of the eight samples to which nucleotide was subsequently added (time 0 to 40 minutes). The "+ nucleotide" values reported in Table 2 are the average and standard deviation of the five time points from 40 to 80 minutes from each sample containing nucleotide. The agreement of these values across nucleotide concentration (300 μM and 2 mM) and nucleotide identity (ADP, AMPPNP, AMPPCP, ATPγS) demonstrates that the anisotropy of the peptide is not affected by the presence of nucleotide. The "+ Hsp104" values reported in Table 1 are the average and standard deviation of the ten time points from 80 to 160 minutes for each nucleotide condition, with the exception of the ATPγS conditions. Because the time courses for ATPγS include an increase in the anisotropy value over the first two anisotropy measurements after addition of protein, the average and standard deviation of the final eight time points (90 to 160 minutes) are reported, representing the final anisotropy of the complex formed by peptide and Hsp104 in the presence of ATPγS. The calculations for average and standard deviation were conducted in the same manner for the Hsp104A503S and ClpB results reported in Supporting Figure 4 and Supporting Tables 3 and 4. The averages and standard deviations for a replicate of the Hsp104 WT anisotropy data reported in Supporting Figure 3 and Table 2 follow the same strategy.

Fluorescence Stopped Flow – Raw Fluorescence

Fluorescence stopped flow experiments were performed using an Applied Photophysics (Leatherhead, U.K.) SX20 stopped-flow fluorimeter as previously described.[16-18] These experiments were conducted at 25 °C in buffer HK150. Fluorescence of the fluorescein on FluNCysRepA50mer was observed by excitation at 494 nm with emission observed above 515 nm using a 515 nm long-pass filter.

The first syringe contained 2 μM Hsp104, 300 μM nucleotide (or no nucleotide), and 20 nM FluNCysRepA50mer (see Figure 3 A). In the motor protein-peptide complex, the fluorescence of the fluorescein is quenched (see Figure S5B and associated description). The second syringe contained 10 mM ATP and 20 μM α-casein. The α-casein serves as a protein trap to bind any free Hsp104 upon mixing, maintaining single-turnover reaction conditions with respect to the Hsp104-polypeptide complex. The contents of each syringe were incubated for one hour at 25 °C prior to the experiment. The change in fluorescence emission was monitored over 400 seconds.

Fluorescence Stopped-Flow – Anisotropy and Total Fluorescence

Fluorescence stopped flow experiments were performed using an Applied Photophysics (Leatherhead, U.K.) SX20 stopped-flow fluorimeter with the fluorescence polarisation accessory, two 515 nm long-pass filters and two R6095 photomultiplier tubes. These experiments were conducted at 25 °C in buffer HK150. FluNCysRepA50mer was excited at 494 nm. The first syringe contained 2 μM Hsp104 and 300 μM ATPγS. The second syringe contained 20 nM FluNCysRepA50mer. The contents of each syringe were incubated for one hour at 25 °C prior to the experiment. Upon rapid mixing, anisotropy and total fluorescence were simultaneously observed for 1800 s.

Additional Results

Analytical Ultracentrifugation Results with Hsp104A503S and ClpB

Sedimentation velocity experiments were performed on the Hsp104 potentiated variant, Hsp104A503S, and the bacterial homologue of Hsp104, *E. coli* ClpB. These experiments were performed identically to the sedimentation velocity experiments described in the main text, with the appropriate protein substitution. The resulting c(s) distributions for Hsp104A503S and ClpB are shown in Supporting Figures 1 and 2, respectively. The resulting weight average sedimentation coefficient for the largest boundaries observed for Hsp104A503S and ClpB are summarized in Supporting Table 1.

Similarly to Hsp104, the predominant c(s) distribution observed for Hsp104A503S in the absence of nucleotide (Supporting Table 1) has a weight average sedimentation coefficient of ~16. S. The dominant c(s) distribution observed for Hsp104A503S in the presence of ADP (blue traces), AMPPNP (green traces), and AMPPCP (orange traces), exhibits a weight average sedimentation coefficient ranging between ~ (15.8 – 16.5) S. By comparison to the analogous experiments with Hsp104, this is consistent with the formation of hydrodynamically similar Hsp104A503S hexamers in the absence of nucleotide as well as in the presence of ADP, AMPPNP, and AMPPCP. In contrast, when the sedimentation velocity experiments are carried out on Hsp104A503S in the presence of ATPγS (red

traces), the weight average sedimentation coefficient of the predominant distribution observed was ~ (17.0 – 17.2) S. This again is consistent with our observations for wild-type Hsp104, suggesting that Hsp104A503S hexamers populated in the presence of ATPγS are hydrodynamically different from those observed in the absence of nucleotide or in the presence of the other nucleotides tested. Notably, the sedimentation coefficient in the presence of 2 mM AMPPNP (dark green trace in Supporting Figure 1 C) is higher than observed in all other conditions except with ATPγS. While visual inspection may suggest that this represents an oligomer consistent with the ATPγS bound oligomer, the sedimentation coefficient is half a Svedberg unit lower than the oligomer observed in the presence of ATPγS indicating that there are still notable differences between the structures. Furthermore, the anisotropy experiments discussed below (Supporting Figure 4) demonstrate that the Hsp104A503S oligomer bound by AMPPNP does not avidly bind peptide.

Interestingly, the predominant c(s) distribution observed for 2 μM ClpB in the absence of nucleotide (Supporting Figure 2 A) was not the largest oligomer in all cases. Here, we use 200 mM NaCl for consistency with our other ClpB experiments, reported here and elsewhere. We have previously shown that at 200 mM NaCl ClpB resides in a distribution of oligomers in which the hexamer is not the predominant species. [7] In order to compare the sedimentation coefficient values for the largest species that could be populated both in the absence and presence of the nucleotides tested, weight average sedimentation coefficient for the boundary with the greatest sedimentation coefficient from each c(s) distribution shown in Supporting Figure 2, was determined and summarized in Supporting Table 1. As with Hsp104 WT and A503S, a hydrodynamically different state for ClpB was observed only in the presence of ATPγS, compared to all other conditions tested.

Reproducibility of anisotropy binding observations

Supporting Figure 3 represents a complete replicate of Figure 2 of the main text. This was accomplished by making up all of the reagents fresh and doing the experiment again on another day. The anisotropy values, with error, are reported in Table 2 of the main text. As can be seen, the data are reproducible.

Potentiated variant Hsp104A503S and bacterial homologue of Hsp104WT *E. coli* ClpB bind peptide avidly only in the presence of ATPγS

Hsp104A503S is a variant with increased disaggregation activity and efficacy against disease models relative to wild type Hsp104.[2,19] We sought to determine whether this enhanced activity was due to differences in polypeptide binding. For example, could Hsp104A503S bind peptide substrate under conditions where Hsp104 could not? To test for this possibility, Hsp104A503S was used in a steady state anisotropy binding experiment identical to that presented in Figure 2 of the main text.

Supporting Figure 4, panel A, shows the anisotropy of the peptide over time. As in the primary text, the experiment begins with each sample containing only FluNCysRepA50mer in buffer HK150. All time points below 40 minutes in Supporting Figure 4A report the

anisotropy of FluNCysRepA50mer in the absence of nucleotide, and also in the absence of Hsp104A503S. The fluorescein modified polypeptide exhibits an anisotropy of $r = 0.072 \pm 0.003$. Next, as shown in Supporting Figure 4, panel A, by an arrow indicating nucleotide addition at 38 minutes, ADP (blue), AMPPNP (green), AMPPCP (orange), or ATPγS (red) was added to each sample such that the final concentration was 300 μM nucleotide (open circles) or 2 mM nucleotide (filled diamonds). As summarized in the top row of Supporting Table 2, the anisotropy measurement for the peptide in the presence of each nucleotide was within one standard deviation of the anisotropy measurement for FluNCysRepA50mer alone.

Hsp104A503S was then added to each sample, as indicated in the figure, to a final concentration of 2 μM. As observed with the wild-type Hsp104 experiments, there was no significant change in the anisotropy of the peptide in the presence of either 300 μM or 2 mM ADP (blue), AMPPNP (green), AMPPCP (orange). As summarized in the bottom row of Supporting Table 2, the anisotropy measurements of the peptide in the presence of Hsp104A503S and ADP, AMPPNP, or AMPPCP were within one standard deviation of each other, and also within one standard deviation of the measurement for FluNCysRepA50mer in the presence of Hsp104A503S with no nucleotide, $r = 0.073 \pm 0.002$. In sharp contrast to the other nucleotides, and consistent with the observations made using wild-type Hsp104, the anisotropy increased to $r = 0.209 \pm 0.004$ in the presence of 300 μM ATPγS, and $r = 0.213 \pm 0.003$ in the presence of 2 mM ATPγS. The increase in anisotropy indicates that FluNCysRepA50mer is bound by the Hsp104A503S oligomer, resulting in the slower tumbling. In comparison to the time course collected for wild-type Hsp104, the magnitude and rate of change in anisotropy upon peptide binding by Hsp104A503S are both greater. This suggests that there may be some subtle differences in binding affinity of the wild-type or variant for this peptide which invite further investigation. The primary observation, however, is that both wild-type Hsp104 and Hsp104A503S require ATPγS in order to avidly bind this model unstructured peptide and the other nucleotides examined cannot substitute.

E. coli ClpB is the bacterial homologue of yeast Hsp104.[20] ClpB and Hsp104 are similar in both structure and function, though Hsp104 is able to disaggregate more complex amyloid aggregates while ClpB cannot.[21] Recent investigation of the molecular mechanism of ClpB revealed that it is a non-processive translocase, in contrast with the prevailing model in the field of "threading" or processive polypeptide translocation.[16,22-24] We sought to determine whether these homologous proteins have the same requirements for peptide binding by performing the anisotropy experiments described above using ClpB.

Supporting Figure 4, panel B, shows the anisotropy of the peptide over time. The experiment begins with each sample containing only FluNCysRepA50mer in buffer HK150. All time points below 40 minutes in Supporting Figure 4B report the anisotropy of FluNCysRepA50mer in the absence of nucleotide, and also in the absence of ClpB. The fluorescein modified polypeptide exhibits an anisotropy of $r = 0.060 \pm 0.003$. Next, as shown in Supporting Figure 4, panel B, by an arrow indicating nucleotide addition at 38 minutes,

ADP (blue), AMPPNP (green), AMPPCP (orange), or ATPγS (red) was added to each sample such that the final concentration was 300 μM nucleotide (open circles) or 2 mM nucleotide (filled diamonds). As summarized in the top row of Supporting Table 3, the anisotropy measurement for the peptide in the presence of each nucleotide was within one standard deviation of the anisotropy measurement for FluNCysRepA50mer alone.

ClpB was then added to each sample, as indicated in Supporting Figure 4, panel B, to a final concentration of 2 μM. As observed with both the wild-type and variant Hsp104 experiments, there was no significant change in the anisotropy of the peptide in the presence of either 300 μM or 2 mM ADP (blue), AMPPNP (green), AMPPCP (orange). As summarized in the bottom row of Supporting Table 3, the anisotropy measurements of the peptide in the presence of ClpB and ADP, AMPPNP, or AMPPCP were within one standard deviation of each other, and also within one standard deviation of the measurement for FluNCysRepA50mer in the presence of Hsp104A503S with no nucleotide, $r = 0.061 \pm 0.002$. In sharp contrast to the other nucleotides, and consistent with the observations made using wild type and variant Hsp104, the anisotropy increased to $r = 0.10 \pm 0.01$ in the presence of 300 μM ATPγS, and $r = 0.133 \pm 0.006$ in the presence of 2 mM ATPγS. Like Hsp104, ClpB only binds peptide in the presence of ATPγS. There are, however, notable differences in the time course for ClpB as compared with the Hsp104 and Hsp104A503S experiments. The increase in anisotropy is slower when the motor protein studied is ClpB. In fact, the 300 μM ATPγS trace (red open circles) appears to still be increasing even 75 minutes after addition of the final component. The time courses generated in the presence of low (300 μM, red open circles) or high (2 mM, red filled diamonds) ATPγS concentrations are notably different. These findings suggest that ClpB binding of the polypeptide substrate is slower, and more dependent upon the ATPγS concentration than its homologue Hsp104.

Binding of FluNCysRepA50mer Results in Quenching of Fluorescein Fluorescence

Nucleotide bound Hsp104 was rapidly mixed with fluorescently modified polypeptide to determine if binding of Hsp104 to polypeptide substrates induces fluorescence changes in addition to the observed anisotropy change. To this end, a solution of Hsp104 and ATPγS was rapidly mixed with a solution of FluNCysRepA50mer as schematized in Supporting Figure 5, panel A; the resulting anisotropy and total fluorescence time courses are displayed in Supporting Figure 5, panel B. The red trace indicates an increase in anisotropy, consistent with the observations in Figure 2. (Note that the L format of the fluorimeter and the T format of the fluorescence stopped-flow give rise to different anisotropy values. In either format, an increase in anisotropy upon binding of Hsp104 to FluNCysRepA50mer is observed.) The simultaneous total fluorescence time course (black trace) shows a decrease in fluorescence, or fluorescence quenching, upon binding. This finding allows us to monitor fluorescence of the pre-bound complex in a translocation assay with an increase in fluorescence indicating dissociation of Hsp104 from polypeptide substrate shown in Figure 3. This is the same approach employed in our studies of the translocation mechanisms of ClpB and ClpA.

Supporting References

(1) Desantis, M. E.; Sweeny, E. A.; Snead, D.; Leung, E. H.; Go, M. S.; Gupta, K.; Wendler, P.; Shorter, J. *J Biol Chem* **2014**, *289*, 848.

(2) Jackrel, M. E.; DeSantis, M. E.; Martinez, B. A.; Castellano, L. M.; Stewart, R. M.; Caldwell, K. A.; Caldwell, G. A.; Shorter, J. *Cell* **2014**, *156*, 170.

(3) Edelhoch, H. *Biochemistry* **1967**, *6*, 1948.

(4) Pace, C. N.; Vajdos, F.; Fee, L.; Grimsley, G.; Gray, T. *Protein Sci* **1995**, *4*, 2411.

(5) Zhao, H.; Ghirlando, R.; Piszczek, G.; Curth, U.; Brautigam, C. A.; Schuck, P. *Analytical biochemistry* **2013**, *437*, 104.

(6) Ghirlando, R.; Balbo, A.; Piszczek, G.; Brown, P. H.; Lewis, M. S.; Brautigam, C. A.; Schuck, P.; Zhao, H. *Analytical biochemistry* **2013**, *440*, 81.

(7) Lin, J.; Lucius, A. L. *Proteins* **2015**, *83*, 2008.

(8) Lin, J.; Lucius, A. L. *Biochemistry* **2016**, *55*, 1758.

(9) Schuck, P. *Biophys J* **1998**, *75*, 1503.

(10) Laue, T. M., Shah, B.D., Ridgeway, T.M., Pelletier, S.L. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science*; S.E. Harding, A. J. R., J.C. Horton, Ed.; Royal Society of Chemistry: Cambridge, 1992.

(11) Cole, J. L. *Biochemistry* **1996**, *35*, 15601.

(12) Veronese, P. K.; Lucius, A. L. *Biochemistry* **2010**, *49*, 9820.

(13) Veronese, P. K.; Stafford, R. P.; Lucius, A. L. *Biochemistry* **2009**, *48*, 9221.

(14) Kuntz, I. D. *J Am Chem Soc* **1971**, *93*, 516.

(15) Li, T.; Lucius, A. L. *Biochemistry* **2013**, *52*, 4941.

(16) Li, T.; Weaver, C. L.; Lin, J.; Duran, E. C.; Miller, J. M.; Lucius, A. L. *The Biochemical journal* **2015**, *470*, 39.

(17) Lucius, A. L.; Miller, J. M.; Rajendar, B. *Methods Enzymol* **2011**, *488*, 239.

(18) Rajendar, B.; Lucius, A. L. *J Mol Biol* **2010**, *399*, 665.

(19) Jackrel, M. E.; Shorter, J. *Dis Model Mech* **2014**, *7*, 1175.

(20) Parsell, D. A.; Sanchez, Y.; Stitzel, J. D.; Lindquist, S. *Nature* **1991**, *353*, 270.

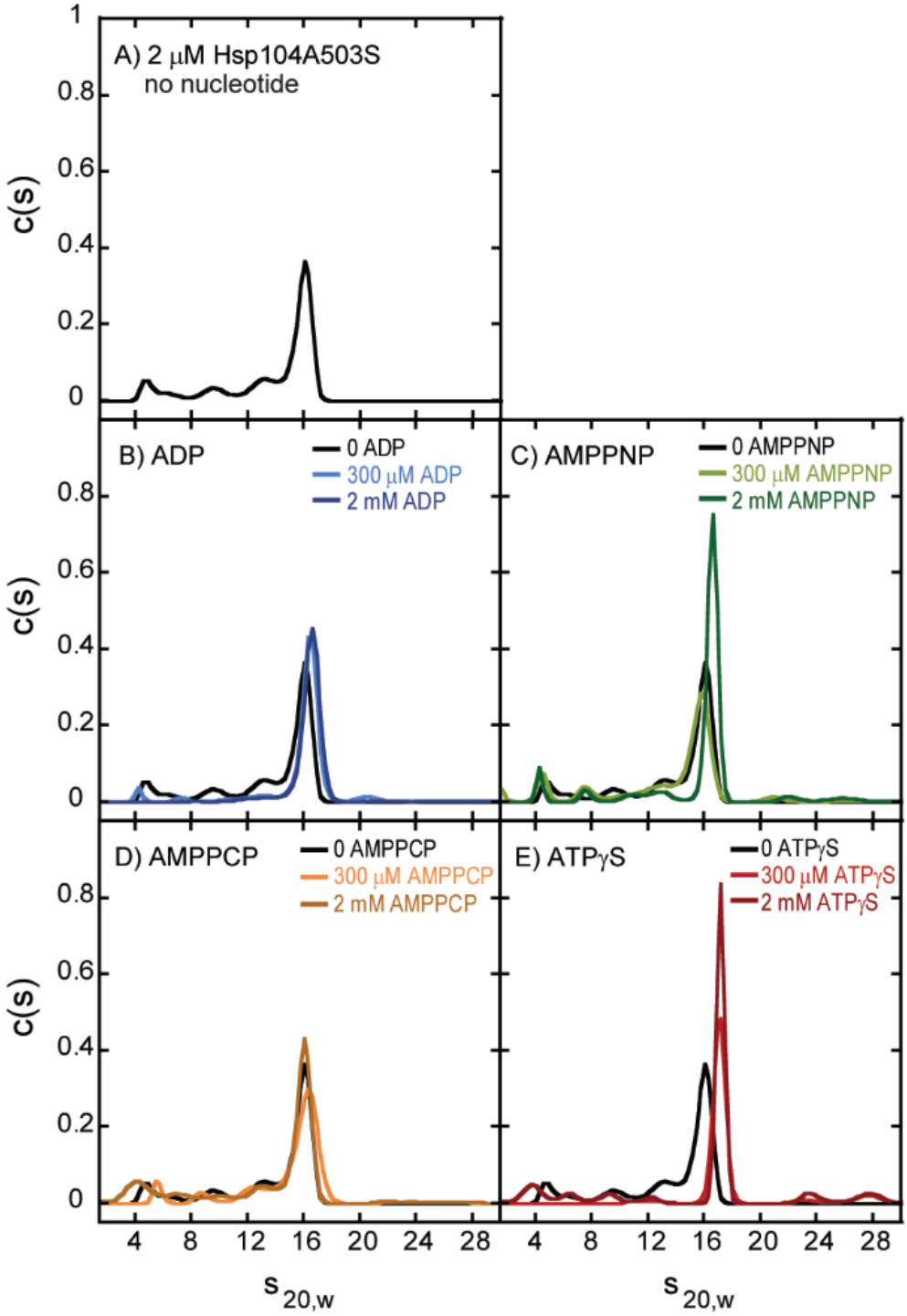
(21) DeSantis, M. E.; Leung, E. H.; Sweeny, E. A.; Jackrel, M. E.; Cushman-Nick, M.; Neuhaus-Follini, A.; Vashist, S.; Sochor, M. A.; Knight, M. N.; Shorter, J. *Cell* **2012**, *151*, 778.

(22) Weibezahn, J.; Tessarz, P.; Schlieker, C.; Zahn, R.; Maglica, Z.; Lee, S.; Zentgraf, H.; Weber-Ban, E. U.; Dougan, D. A.; Tsai, F. T.; Mogk, A.; Bukau, B. *Cell* **2004**, *119*, 653.

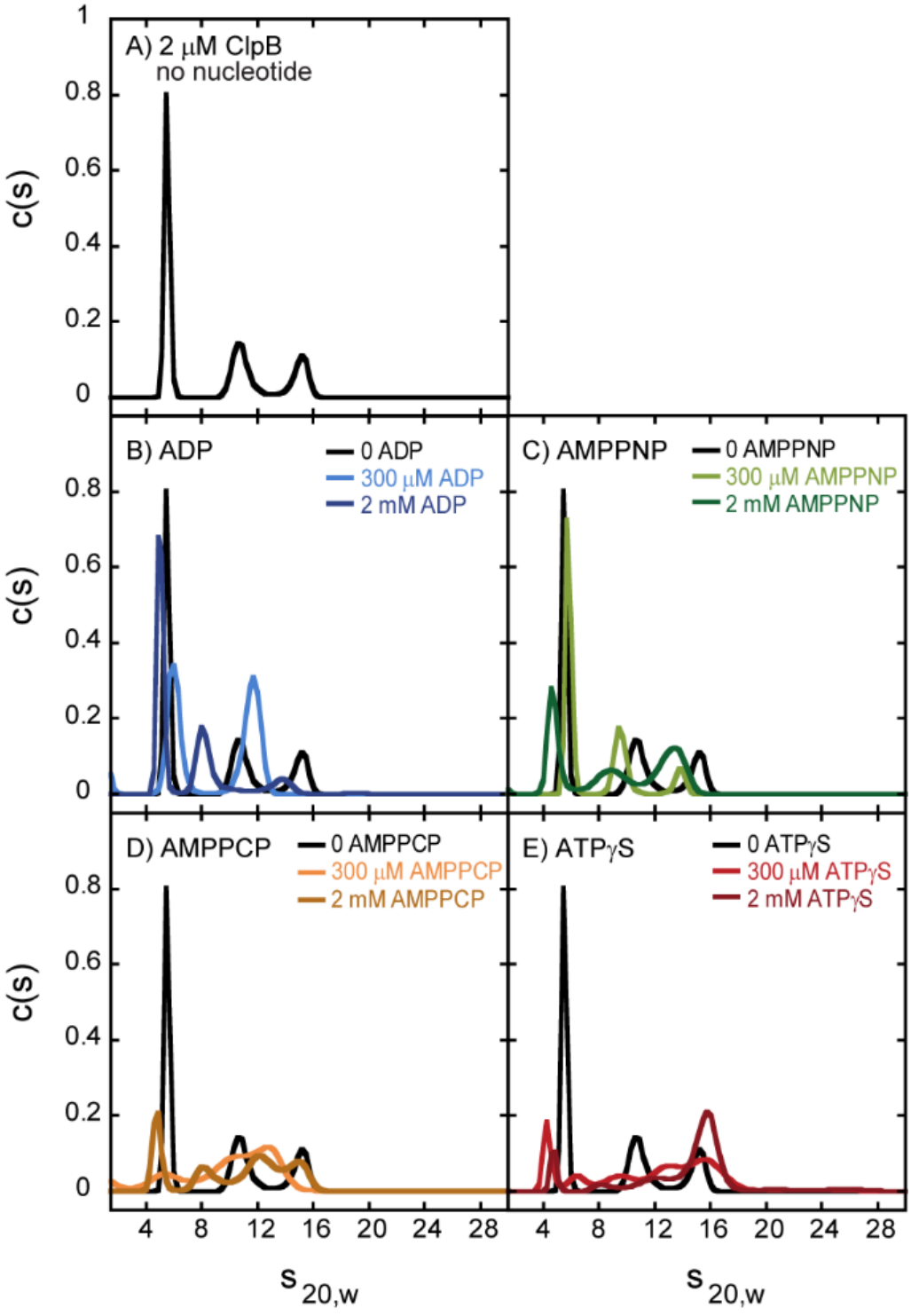
(23) Watanabe, Y. H.; Nakazaki, Y.; Suno, R.; Yoshida, M. *The Biochemical journal* **2009**, *421*, 71.

(24) Nakazaki, Y.; Watanabe, Y. H. *Genes Cells* **2014**, *19*, 891.

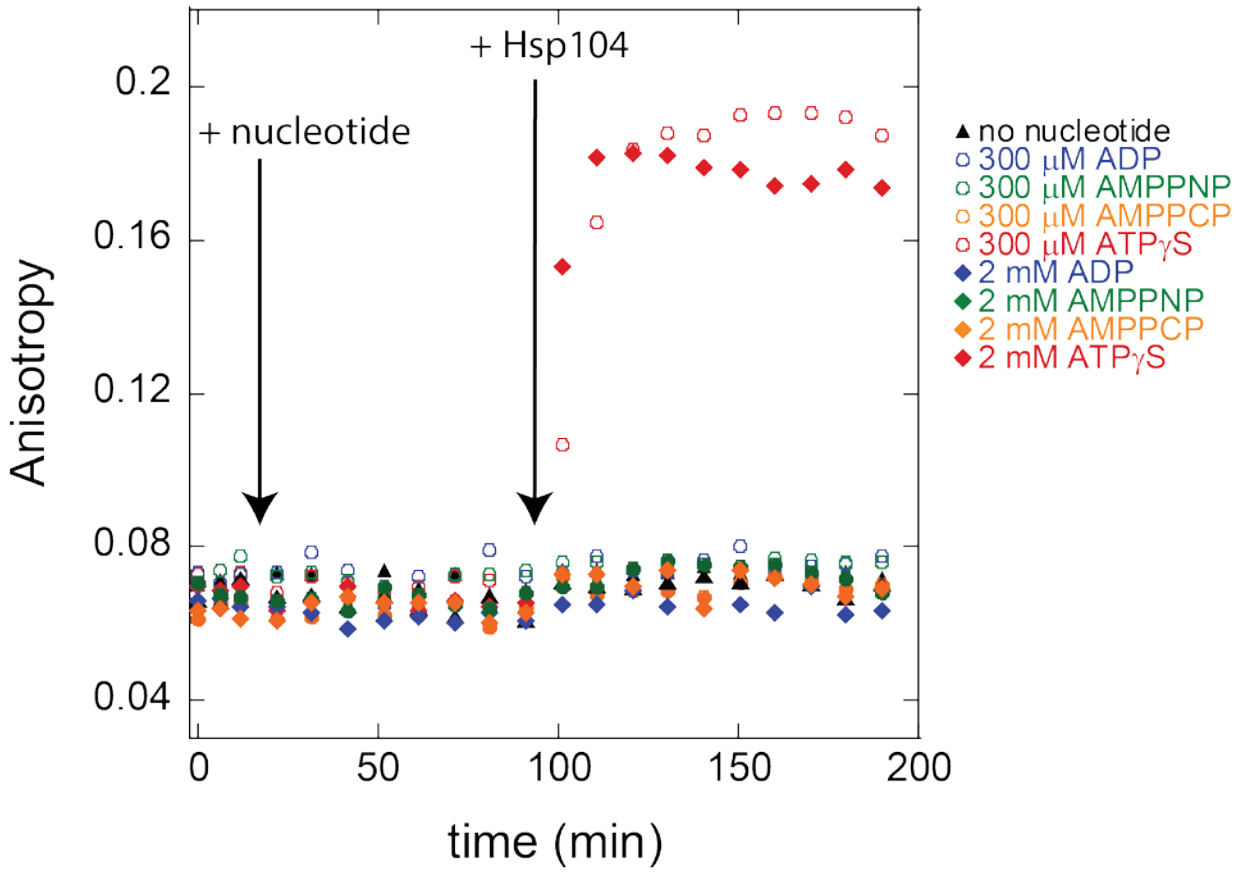
Supporting Figures



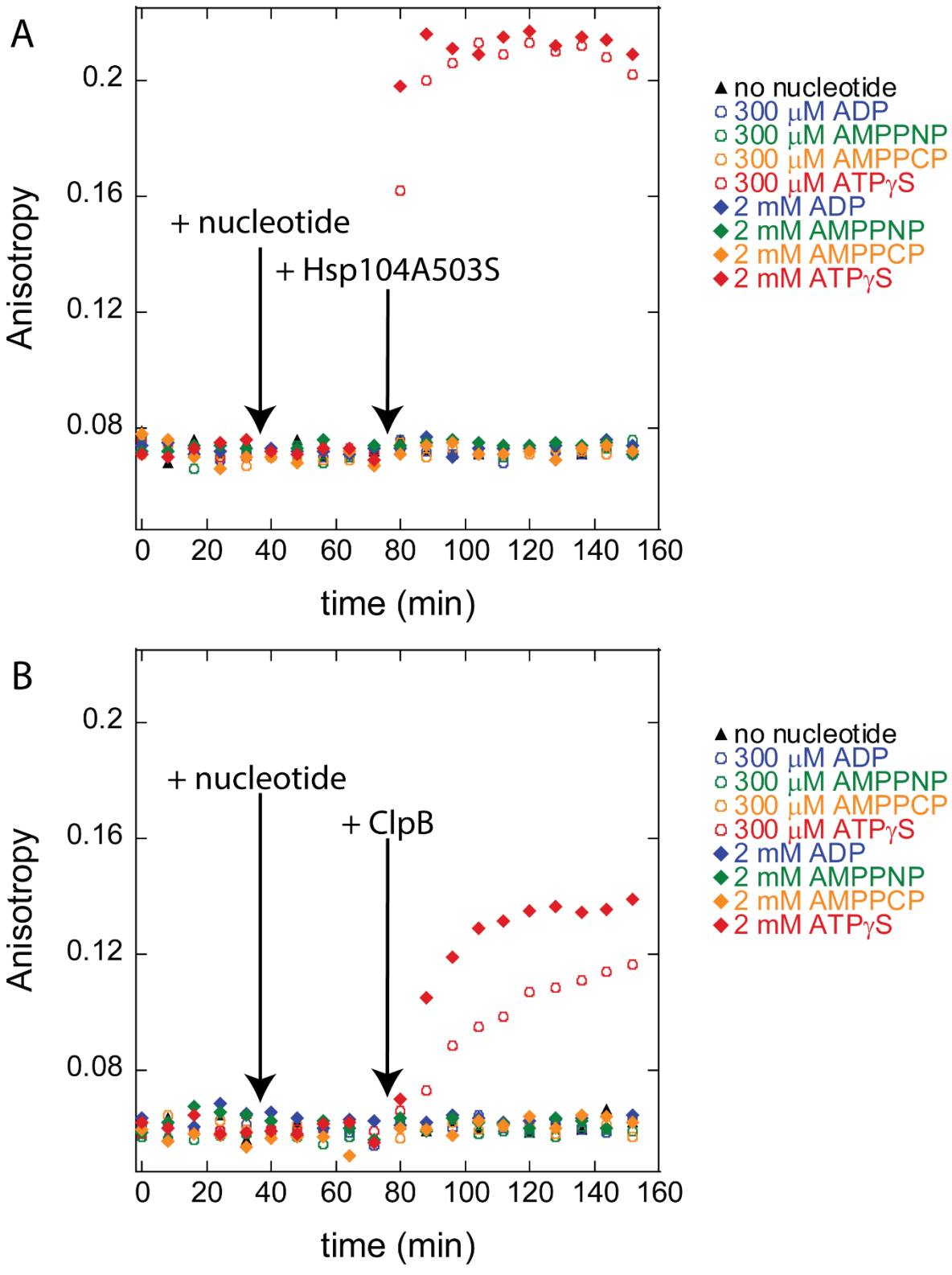
Supporting Figure 1. Sedimentation velocity c(s) distributions of Hsp104A503S in the absence and presence of nucleotides. Sedimentation velocity experiments were performed on 2 μM Hsp104 in the absence (A) and presence of ADP, AMPPNP, AMPPCP, or ATPγS (B-E).



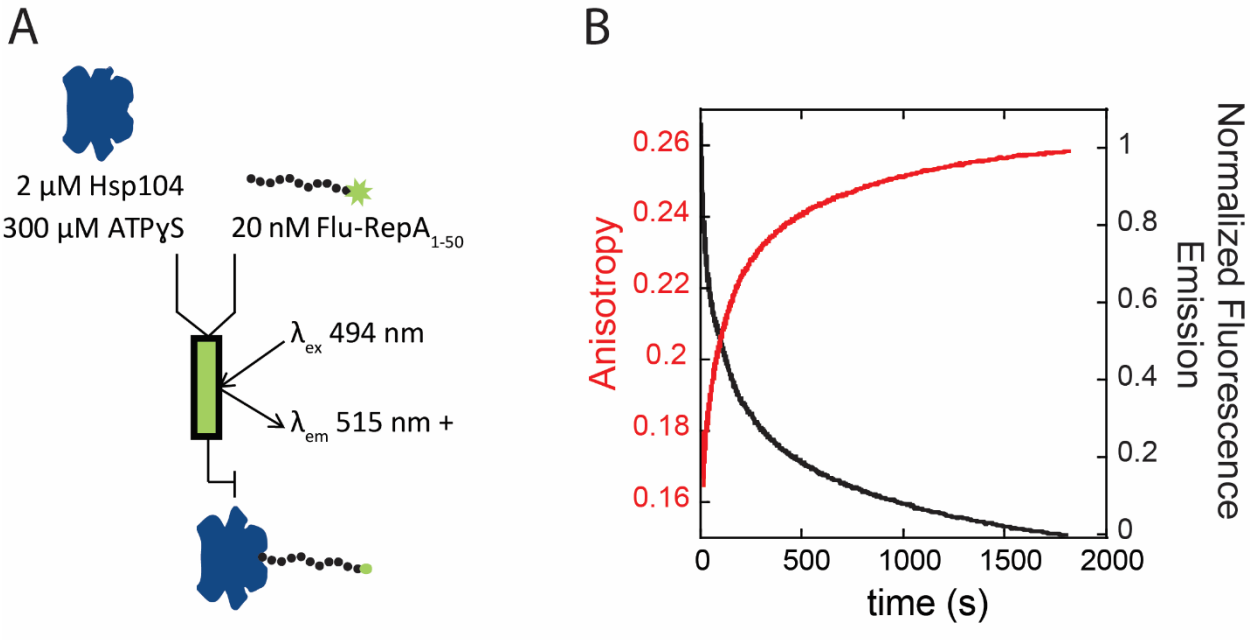
Supporting Figure 2. Sedimentation velocity c(s) distributions of ClpB in the absence and presence of nucleotides. Sedimentation velocity experiments were performed on 2 μM ClpB in the absence (A) and presence of ADP, AMPPNP, AMPPCP, or ATPγS (B-E).



Supporting Figure 3. Representative replicate of fluorescence anisotropy measurements of Flu-NCysRepA50mer before and after subsequent additions of nucleotide and Hsp104 where indicated.



Supporting Figure 4. Fluorescence anisotropy measurements of Flu-NCysRepA50mer before and after subsequent additions of nucleotide and (A) Hsp104A503S or (B) ClpB where indicated.



Supporting Figure 5. Binding experiment reaction schematic (A) and fluorescence anisotropy and total fluorescence time courses (B). Hsp104 and ATPγS were rapidly mixed with Flu-NCysRepA50mer. Fluorescence anisotropy and total fluorescence were observed simultaneously.

Supporting Table 1. Hsp104A503S and ClpB largest c(s) distribution

Condition	Hsp104A503S $s_{20,w}$ (S)	ClpB $s_{20,w}$ (S)
No nucleotide	16.014 ± 0.005	15.16 ± 0.02
300 μM ADP	16.414 ± 0.003	11.89 ± 0.01
2 mM ADP	16.465 ± 0.003	13.74 ± 0.03
300 μM AMPPNP	15.749 ± 0.004	13.86 ± 0.03
2 mM AMPPNP	15.930 ± 0.004	13.27 ± 0.01
300 μM AMPPCP	16.029 ± 0.008	12.98 ± 0.01
2 mM AMPPCP	15.930 ± 0.004	14.45 ± 0.01
300 μM ATPγS	17.041 ± 0.003	15.96 ± 0.01
2 mM ATPγS	17.165 ± 0.003	15.848 ± 0.004

Standard deviations were determined from Monte Carlo analysis in SedFit.

Supporting Table 2. Fluorescence anisotropy of FluNCysRepA50mer with Hsp104A503S

	ADP	AMPPNP	AMPPCP	ATPγS	ADP	AMPPNP	AMPPCP	ATPγS
	300 μM	300 μM	300 μM	300 μM	2 mM	2 mM	2 mM	2 mM
+ nucleotide	0.0708 ± 0.0007	0.071 ± 0.002	0.0695 ± 0.0008	0.0720 ± 0.0006	0.072 ± 0.001	0.073 ± 0.002	0.070 ± 0.003	0.071 ± 0.002
+ Hsp104 A503S	0.072 ± 0.002	0.073 ± 0.002	0.072 ± 0.001	0.209 ± 0.004	0.074 ± 0.002	0.074 ± 0.001	0.072 ± 0.002	0.213 ± 0.003

The averages and standard deviations were calculated as described in Experimental Procedures.

Supporting Table 3. Fluorescence anisotropy of FluNCysRepA50mer with ClpB

	ADP	AMPPNP	AMPPCP	ATPγS	ADP	AMPPNP	AMPPCP	ATPγS
	300 μM	300 μM	300 μM	300 μM	2 mM	2 mM	2 mM	2 mM
+ nucleotide	0.058 ± 0.003	0.057 ± 0.002	0.059 ± 0.002	0.060 ± 0.001	0.063 ± 0.002	0.060 ± 0.003	0.055 ± 0.003	0.059 ± 0.003
+ ClpB	0.061 ± 0.002	0.060 ± 0.002	0.060 ± 0.001	0.10 ± 0.01	0.063 ± 0.001	0.063 ± 0.002	0.062 ± 0.002	0.133 ± 0.006

The averages and standard deviations were calculated as described in Experimental Procedures.